BACTERIAL SYMBIONTS OF INSECT PATHOGENIC NEMATODES OF THE FAMILIES STEINERNEMATIDAE AND HETERORHABDITIDAE

by

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There is no material contained in this thesis which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief this thesis contains no copy or paraphrase of material previously published or written by another person, except when due reference has been made in the text of the thesis.

CONTENTS

SUMMARY

Insect pathogenic nematodes of the families Steinermatidae and Heterorhabditidae from 42 populations (four genera and over nine species) from Australasia, Europe and North America were found to be symbiotically associated with bacteria of the genus *Xenorhabdus*.

A taxonomic study of *Xenorhabdus* showed that the bacterial symbionts of the Heterorhabditidae were all *X. luminescens* and those of the Steinernematidae, with one possible exception, were all *X. nematophilus*. Numerical analysis of the taxonomic data indicated that the genus might be heterogeneous.

Although the symbionts of most of the Steinernematidae (including *Steinernema kraussei* which had previously been reported to be associated with a *Flavobacterium* sp.) were classified within one species, the differences between the bacteria associated with the various steinernematid species were great enough to warrant the erection of four subspecies.

Results obtained with some tests used in the taxonomic study differed from those previously reported. Amended descriptions of the genus *Xenorhabdus* and its two species are proposed.

Each *Xenorhabdus* species was found to produce two forms of colony. One form, designated the primary form, promoted significantly greater nematode fecundity than did the secondary; it produced antimicrobial substances whereas the secondary did not; and it was the form usually found in the infective stage nematodes. The primary form was unstable under many conditions, resulting in production of the secondary form which in two subspecies was also unstable reverting to the primary form. Attempts were made to elucidate the mechanism determining the change of form.

The primary form of each *Xenorhabdus* sp. had a wide spectrum of antimicrobial activity, inhibiting both yeasts and bacteria. All *Xenorhabdus* isolates tested were sensitive to some other *Xenorhabdus* isolates and some were mutually inhibitory. Each *Xenorhabdus* isolate apparently produces more than one antimicrobial agent.

The specificity of the nematode/bacterium associations was also tested. The nematodes were usually able to reproduce when cultured with the symbiont of another *Neoaplectana* sp. but never with the symbiont of a *Heterorhabditis* sp. or that of an undescribed steinernematid. Although infective juveniles were equally able to carry within their intestines bacteria isolated from all other strains of the same species, only a small proportion was able to carry *X. nematophilus* isolated from another species.

Most of the *Xenorhabdus* isolates tested were highly pathogenic when injected intrahaemocoelically into *Galleria mellonella* larvae (LD_{50} < 20 cells). However, even a small dose of a poorly pathogenic isolate was sufficient to kill *G. mellonella* when injected with its nematode associate which was unable to kill *G. mellonella* when injected alone.

Xenorhabdus species were found to overwhelmingly dominate the bacterial flora of nematode-infected *G. mellonella* while the nematodes were maturing and reproducing. As the new generation of infective juveniles was produced, the proportion of *Xenorhabdus* in the flora declined. Secondary form *X. nematophilus* was unable to dominate the flora as effectively as the primary form.

INTRODUCTION

Over the past 10-15 years there has been an increasing interest in the possibility of using nematodes for the control of insect pests. A group of nematodes, comprised of the genera *Steinernema*, *Neoaplectana* and *Heterorhabditis*, has attracted particular attention because of their extremely wide host range (Poinar 1979).

During the 1930's and 1940's one species of this group, *Neoaplectana glaseri*, was released over a large area for the control of Japanese beetle in the USA, being replaced by the milky disease organism, *Bacillus popilliae*, for economic reasons. However, methods have recently been introduced that have dramatically reduced the cost of production of *N. glaseri* and related nematodes by vastly increasing their rate of production (Bedding 1976, 1981), leading to their possible commercial utilization against sugar cane and pasture scarab larvae. *Neoaplectana bibionis* is already being used for the control of *Synanthedon tipuliformis* in blackcurrants (Bedding & Miller 1981a, Miller & Bedding 1982) while *Heterorhabditis heliothidis* is used for the control of *Otiorhynchus sulcatus* in potted plants (Bedding & Miller 1981b; Simons 1981).

A characteristic of these nematodes is their association with insect pathogenic bacteria. The association is considered by most workers to be mutualistic although Lysenko (1981) considers that the association is of doubtful importance. Some details of the interaction between one nematode species, *Neoaplectana feltiae* (syn. *Neoaplectana carpocapsae*, Stanuszek 1974) and its bacterial associate have been described (Dutky 1959; Poinar 1966; Poinar & Thomas 1966, 1967; Götz *et al.* 1981) as has the taxonomy of a few of the bacterial associates (Parvez 1974; Thomas & Poinar 1979).

The aim of this study was to examine the nematode/bacterium associations present in a range of nematode genera and species, with emphasis on the significance of the association and the taxonomic relationships between the bacteria.

LITERATURE REVIEW

Early descriptions of steinernematid nematodes by Steiner (1923, 1929) (*Steinernema kraussei, N. glaseri*), Travassos (1932) (*Neoaplectana menozzii*), Filipjev (1934) (*N. feltiae*) and Bovien (1937) (*Neoaplectana affinis*) failed to recognise their specific association with bacteria. Such associations were first reported by Dutky (1937) for *N. feltiae* (= *N. carpocapsae*) and Bovien (1937) for *N. bibionis*. Several descriptions of *Neoaplectana* species were published subsequently without reference to associated bacteria (Glaser *et al.* 1942; Hoy 1954; Weiser 1955, 1958; Weiser & Köhler 1955; Kirjanova & Puchkova 1955; Kakulya & Veremchuk 1965; Artyukhovsky 1967; Veremchuk 1969; Turco 1970; Veremchuk & Litvinchuk 1971). Although the significance of the association between *N. feltiae* and its symbiont was indicated by Dutky (1959), it was not until Poinar & Thomas (1966) demonstrated the significance of this association that other workers recorded similar associations between other nematode species and bacteria.

Specific associations between insect pathogenic nematodes and bacteria have now been recorded for *N. bibionis* (Bovien 1937), *N. feltiae* (Dutky 1937), *N. glaseri* (Poinar & Brooks 1977), and undescribed *Neoaplectana* sp. (Thomas & Poinar 1979), *Heterorhabditis bacteriophora* (Poinar 1975), *Heterorhabditis heliothidis* (Khan *et al.* 1976), an undescribed *Heterorhabditis* sp. (Thomas & Poinar 1979) and *S. kraussei* (Mráček 1977). Comments made by those describing other species of these nematodes (Poinar 1979) indicate that it is very likely that nematodes of all known species of Steinernematidae and Heterorhabditidae are associated with specific bacteria.

Significance of the Nematode/Bacterium Association

The life cycles of all the Steinernematidae and Heterorhabditidae and their interactions with their bacterial symbionts appear to be similar to those described for *N. feltiae* (= *N. carpocapsae*) (Poinar 1979). The Heterorhabditidae differ significantly from the Steinernematidae in that infective stage steinernematids mature into males or females within the host while infective stage heterorhabditids become hermaphroditic adults. In both families adults of the second generation are either male or female.

The infective stage nematode is a free-living, non-feeding juvenile that normally inhabits the soil. Poinar & Thomas (1966) found that this stage of *N. feltiae* carries only one species of bacterium within its intestine. In infective stage *N. feltiae* the symbiont is restricted to the ventricular portion of the intestine (Poinar 1967; Poinar & Leutenegger 1968) while in *N. bibionis* it is further restricted to a vesicle in the ventricular portion of the intestine (Bovien 1937). Poinar *et al.* (1977) found bacterial cells "in the ventricular portion and in the intestine proper" of infective stage *H. bacteriophora* and Wouts (1979) reported that the bacteria were restricted to the anterior portion of the intestine of *H. heliothidis* infective stage juveniles. Kaya & Brayton (1978) and Kaya (1980) demonstrated that infective stage *N. feltiae* may also harbour viable granulosis virus within the intestine.

The infective stage nematode is attracted to an insect host (Bedding & Akhurst 1975) and enters via mouth, anus or spiracles (Poinar & Himsworth 1967; Sandner & Stanuszek 1971; Georgis & Hague 1979). The

nematode penetrates to the haemocoel where it releases the symbiotic bacteria which proliferate causing a septicaemia that kills the insect (Poinar 1966). Insect pathogenicity is not only attributable to the bacterial symbiont; axenic infective stages of *N. feltiae* are able to kill axenic larvae of the wax moth *Galleria mellonella* (Poinar & Thomas 1966) and diapausing pupae of *Hyalophora cecropia* (Götz *et al.* 1981). Sandner *et al.* (1977) showed, for several insect species, an inverse relationship between the number of *N. feltiae* applied and the time taken to kill the insects.

Seryczynska & Kamionek (1974) and Seryczynska (1976) showed that although *Leptinotarsa decemlineata* larvae infected with *N. feltiae* or injected with its bacterial symbiont responded with an increase in the number of free haemocytes, the larvae died. Götz *et al.* (1981) showed *N. feltiae* protects its symbiont by degrading the antibacterial proteins of the diapause pupa of the giant silk moth, *Hyalophora cecropia*.

Dutky (1959) claimed that the bacterial symbiont of *N. feltiae* serves as food for the nematodes and produces an antibiotic that prevents putrefaction of the cadaver by other microorganisms. Poinar & Thomas (1966) showed that, for reproduction, *N. feltiae* requires the presence of its symbiont, or a suitable substitute bacterium, in the haemolymph; *N. feltiae* were able to reproduce in axenic *Galleria* larvae in the presence of the symbiont or of *Pseudomonas aeruginosa* though not of *Bacillus cereus*, *Serratia marcescens* or in the absence of bacteria. The fecundity of the nematodes was much less when *Ps. aeruginosa* rather than the symbiont was present.

After one, two or occasionally three reproductive cycles, a new generation of infective stage nematodes is produced. These infective stage nematodes may remain within the cadaver for several months and leave when the cadaver is in contact with free water (Dutky 1959). The symbiotic bacterium does not survive well independently of the nematode in soil or water (Poinar 1979) and is not pathogenic for insects when administered *per os* (Poinar & Thomas 1967; Milstead 1979a); hence the bacterium must rely on the nematode to transfer it from the cadaver to the haemocoel of a new host.

The bacterial symbionts of *Heterorhabditis* spp. are luminescent (Poinar *et al.* 1977; Khan & Brooks 1977; Thomas & Poinar 1979). Poinar *et al.* (1980) suggested that this might aid the nematodes in locating a new host if healthy insects, or other organisms that would facilitate the distribution of the nematodes, were attracted to the glowing cadavers.

Although almost all workers have accepted the importance of the bacterial symbiont for these nematodes, Lysenko & Weiser (1974) and Lysenko (1981) do not. Lysenko & Weiser (1974) were unable to isolate a bacterium from *N. feltiae*, or from *Galleria* larvae infected with *N. feltiae*, corresponding to the description of Poinar *et al.* (1971). They concluded that the nematodes did not merely act as "living syringes" and that other factors were involved.

Taxonomy of the Steinernematidae and Heterorhabditidae

The family Steinernematidae consists of two genera: *Steinernema* and *Neoaplectana*. However, Bedding (in press) and Wouts *et al.* (1982) consider that *Neoaplectana* is a junior synonym of *Steinernema*.

The genus *Steinernema* contains one described species, *S. kraussei* and at least one other (Mráček 1980).

While Turco *et al.* (1971) recognised 13 species in the genus *Neoaplectana*, Poinar (1979) recognised only seven. However, of these, *N. feltiae* and *N. carpocapsae* were considered by Stanuszek (1974) and Wouts *et al.* (1982) to be synonymous. Both Poinar (1979) and Stanuszek (1974) used hybridisation studies to confirm some of their conclusions based on morphological examination. However, as there were no live nematodes of populations from which some species were described, the different views have not been resolved[a].

Four species have been described in the only genus of the family Heterorhabditidae (Poinar 1975; Khan *et al.* 1976; Poinar 1979). Nematodes of this genus have now been discovered in many parts of the world (Wouts 1979; Bedding & Miller 1981b; Simons 1981; Sexton & Williams 1981; Stanuszek pers. comm.). The taxonomy of this family is not well understood and workers have generally refrained from establishing new species.

Taxonomy of Bacteria Symbiotically Associated with the Steinernematidae and Heterorhabditidae

Poinar & Thomas (1965) characterized the symbiont of the DD136 strain of *N. feltiae* describing it as a new species, *Achromobacter nematophilus*. Poinar *et al.* (1971) extended this characterisation and showed that the symbiont of the Agriotos strain of *N. feltiae* was very

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[a] The synonmy of *N. feltiae* and *N. carpocapsae* has been accepted in this thesis.

similar. However, following a recommendation of Hendrie *et al.* (1974), the genus *Achromobacter* was rejected[a] (Bergey's Manual of Determinative Bacteriology, 1974) and the species became *incertae sedis*. The bacterial symbionts of *H. bacteriophora* and *H. heliothidis* were characterised, but not named, by Poinar *et al.* (1977) and Khan & Brooks (1977) respectively. Subsequently, Thomas & Poinar (1979) erected a new genus, *Xenorhabdus*, within the family Enterobacteriaceae to accommodate the symbionts of *Neoaplectana* and *Heterorhabditis*. They described two species in this genus: *Xenorhabdus nematophilus* (symbiotic with *N. feltiae*, *N. bibionis* and some undescribed *Neoaplectana* sp.) and *Xenorhabdus luminescens* (symbiotic with *Heterorhabditis* spp.).

X. luminescens is the only terrestial luminous bacterium that has been reported (Nealson & Hastings 1979). Poinar *et al.* (1980) found that the enzyme that catalyzes light emission in *X. luminscens* is a typical bacterial luciferase.

Parvez (1974) showed that the *X. nematophilus* symbionts of the DD136, Agriotos and Mexican strains of *N. feltiae* were antigenically similar, though not identical, and different from the symbionts of *N. glaseri* and *N. bibionis*.

Mráček (1977) consistently isolated a *Flavobacterium* sp. from sawflies infected with *S. kraussei*. He concluded that the association between *S. kraussei* and the *Flavobacterium* sp. was similar to that between *N. feltiae* and *X. nematophilus*.

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[a] The name *Achromobacter* was revived (Yabuuchi & Tano, 1981) after the genus *Xenorhabdus* was described. There is, however, no conflict between the genera; the new genus *Achromobacter* is restricted to aerobic and nonfermentative bacteria.

Production of Antibacterial Agents by Xenorhabdus spp.

Kirjanova & Puchkova (1955) suggested that *Neoaplectana bothynoderi* (syn. *N. feltiae*; Wouts *et al.* 1982) release substances that inhibit decomposition of infected beet weevil larvae; they were, however, unaware of a nematode/bacterium association.

Dutky (1959) stated, without producing evidence, that the bacterial symbiont of the DD136 strain of *N. feltiae* elaborated an antibiotic that prevents putrefaction of the cadaver and later (Dutky 1974) specified it to be a wide spectrum antibiotic. Poinar, Hess & Thomas (1980) found that chloroform inactivated colonies of *X. nematophilus* and *X. luminescens* on nutrient agar inhibited the growth of *B. cereus* subsp. *mycoides* and *Bacillus subtilis* in a soft agar overlay. The *Xenorhabdus* cultures contained phage tail-like particles that were able to adsorb to *B. cereus* cells. Poinar, Hess & Thomas (1980) identified the particles as defective bacteriophages and concluded that they were identical with the bactericidal agent.

Paul *et al.* (1981) found variations in the spectra of antibacterial activity of nine strains of *X. nematophilus* and *X. luminescens* tested against *Vibrio* spp. and *Photobacterium* spp. and isolated antibacterial compounds from an *X. nematophilus* and an *X. luminescens* isolate. The four antibacterial compounds isolated from *X. nematophilus* were acetoxyl indoles while the two isolated from *X. luminescens* were stilbenes which are not commonly found in bacteria. The origins of the two groups of antibacterial compounds probably differ considerably; acetoxyl indoles are thought to be degradation products of tryptophan, at least in plants, while stilbenes may be derived via polyketide biosynthesis (Paul *et al.* 1981).

Specificity of the Nematode/Bacterium Association

Poinar & Thomas (1966) showed that *N. feltiae* was able to reproduce in axenic *Galleria* either with its symbiont or with *Ps. aeruginosa* but not with *B. cereus* or *S. marcescens*. Poinar (1979) reported that *N. glaseri* reproduced in monoxenic culture with *X. nematophilus* isolated from *N. feltiae* or with *Alcaligenes faecalis, Proteus rettgeri* or *Ps. aeruginosa* but not with *S. marcescens*. However, none of the infective stage *N. glaseri* produced in these cultures retained bacteria in the intestine.

Pathogenicity of Xenorhabdus spp.

The LD50 for most facultative, non-spore-forming insect pathogens injected into the haemocoel of lepidopterous larvae is in the range 5-100 cells (Lysenko 1981).

Poinar & Thomas (1967) found that a dosage of 1-3 cells of the symbiont of *N. feltiae* injected into the haemocoel of *Galleria* larvae was lethal. This finding is supported by Lysenko & Weiser's (1974) estimation of one cell as the LD50 for this bacterium injected intrahaemocoelically into *Galleria* larvae. Milstead (1979a) found that less than 15 cells of *X. luminescens* per insect killed 100% of *Galleria* larvae injected intrahaemocoelically. Neither bacterium was pathogenic when applied topically or *per os* (Poinar & Thomas 1967; Milstead 1979b). When Sandner *et al.* (1977) injected *L. decemlineata* larvae *per anus* with *X. nematophilus* up to 30% of the insects died. However, 25% of larvae injected *per anus* with physiological saline also died. It therefore seems likely that death was due to puncturing of the intestine by the cannula with consequent introduction of bacteria to the haemocoel.

Götz *et al.* (1981) found that the LD50 of *X. nematophilus* injected into diapausing *H. cecropia* pupae was much higher than that reported for *Galleria* larvae; the LD50 for normal pupae was ca. 500 cells and for "immunised" pupae (previously injected with *Enterobacter cloacae*) was ca. 500,000.

Sandner *et al.* (1977) demonstrated that *X. nematophilus* produced a heat stable endotoxin that killed *Galleria* and *L. decemlineata* larvae when injected into the haemocoel.

The effect of *Xenorhabdus* on insects prior to death has been examined by two groups. Sandner *et al.* (1977) reported that *Galleria* and *L. decemlineata* larvae injected with *X. nematophilus* responded with an initial increase in the number of free haemocytes followed by a decline below the normal level. Milstead (1980a,b) reported decreases in silk production by *Galleria* and in the feeding rate, wet weight and frass production of seventh instar *Schizura concinna* injected with *X. luminescens*. Milstead (1979a) found no significant reduction in haemolymph refractive index in *Galleria* injected with *X. luminescens* and concluded that *X. luminescens* reduced haemolymph solids to a minimal extent.

Gaugler & Boush (1979) found that intraperitonal and *per os* inoculation of rats with infective stage *N. feltiae* produced no signs of pathogenicity, toxicity, infection or nematode-related histopathology. Poinar *et al.* (1982) also found that mice were not noticeably affected by subcutaneous injection of infective stage *N. feltiae* or *H. bacteriophora* or by subcutaneous or intracerebral injections of the symbiotic bacteria. Obendorf *et al.* (in press) tested the

susceptibility of mammals to *X. nematophilus* isolated from *N. bibionis*; when rats, mice, guinea pigs and rabbits were inoculated with the bacterium *per os*, by intradermal, subcutaneous and/or intraperitoneal injection, by inhalation, by skin contact or conjunctival application, no evidence of infectivity, pathogenicity or toxicity was detected and *X. nematophilus* could not be re-isolated from the treated animals.

MATERIALS AND METHODS

GENERAL

Nematodes

The sources of nematodes are listed in Table 1.

N. glaseri and the Agriotos strain of *N. feltiae* (syn. *N. carpocapsae*) were identified by Prof. G.O. Poinar, University of California, Berkeley. Dr. R.A. Bedding, CSIRO, Hobart confirmed these identifications and identified a Tasmanian isolate, T231, as *N. bibionis*. Because of the confused state of the taxonomy of Steinernematidae, other *Neoaplectana* isolates were identified to species level by a cross-breeding technique. The description of this technique has already been published and is included in Appendix I.

S. kraussei was identified by Dr. J. Weiser, C.S.A.V., Prague and supplied by Dr. Z. Mráček, C.S.A.V., Ceske Budejovice, as an *in vitro* monoxenic culture. When doubt was raised about the significance of the bacterium originally supplied and the culture was lost, Dr. Mráček kindly re-isolated this species from the field and supplied infective juveniles. Dr. R.A. Bedding confirmed that the infective juveniles were of the same species as the nematodes originally supplied in *in vitro* culture.

H. bacteriophora and the North Carolina strain of *H. heliothidis* used in this study were the type strains of these species. The New Zealand strain of *H. heliothidis* was identified by Dr. W. Wouts, D.S.I.R., Auckland.

All nematode strains were cultured *in vivo* in *G. mellonella* larvae as described by Poinar (1979) for *"N. carpocapsae"*. Some strains were also cultured monoxenically with their respective symbionts on nutrient

Table 1. Sources of nematodes and their associated bacteria.

Nematode Isolate	Source	Associated Bacteria Isolate	Source[a]
Heterorhabditis bacteriophora	W. Wouts, DSIR New Zealand	B/1	I
		B/2	M
H. heliothidis			
Nth. Carolina strain	W. Wouts, DSIR, New Zealand	C/1	I
		C/2	M
N.Z. strain	W. Wouts, DSIR, New Zealand	NZH	I
Heterorhabditis sp.			
	Darwin, Northern Territory	D/1	I
		D/2	M
Polish strain	S. Stanuszek, Institute of Ecology, Warsaw, Poland	HP/1	I
		HP/2	G
	Yepoon, Queensland	Q380	I
	Graphognathus leucoloma larva, Geelong, Victoria	V16	I
	Wynyard, Tasmania[b]	T280/1	I
		T280/2	M
	Bruny Island, Tasmania[b]	T301	I
	Sandy Bay, Tasmania[b]	T310	I
	Devonport, Tasmania	T327	I
Neoaplectana bibionis			
Czech strain	Z. Mracek, C.S.A.V., Czechoslovakia	NBC	I
	Murrumbateman, N.S.W.[b]	N51	I
	Black Mountain, A.C.T.[b]	N60	I
New Zealand strain	W. Wouts, DSIR, New Zealand	NZ	I
	Dover, Tasmania[b]	T228	I
	Risdon Vale, Tasmania[b]	T231/1	I
		T231/2	G
	Dover, Tasmania[b]	T268	I
	Nive River, Tasmania[b]	T292	I
	Plenty, Tasmania[b]	T298	I
	Bruny Island, Tasmania[b]	T302	I
	Bruny Island, Tasmania[b]	T307	I
	Mt. Wellington, Tasmania[b]	T319/1	I
		T319/2	G
	Otiorhynchus sulcatus larva, Nicholls Rivulet, Tasmania[b]	T335/1	I
		T335/2	M
Neoaplectana bibionis	S. Sexton, P.R.I., Burnley Victoria.	V1	I
		V3	I
Neoaplectana feltiae			
Agriotos strain	G.O. Poinar, University of California, Berkeley, U.S.A.	A23	M
		A25	G
DD136 strain	Murrumbateman, N.S.W.[b]	AN/5	ATCC 19061
		N55	I
Pieridarum strain	S. Stanuszek, Institute of Ecology, Warsaw, Poland.	P1	I
	Vespula sp., Mt. Nelson, Tasmania	TN6	I
	Powranna, Tasmania[b]	TP7	I
Neoaplectana glaseri	H. Kaya, University of California, Davis, U.S.A.	G/1	I
		G/2	M
Neoaplectana species M	Mackay, Queensland[b]	Q58/1	I
		Q58/2	M
	Yepoon, Queensland[b]	Q385/1	I
		Q385/2	M
	Bowenia S.F., Queensland[b]	Q393/1	I
		Q393/2	G
	Tonganah, Tasmania[b]	T80	I
	Cleveland, Tasmania[b]	T171	I
	Bruny Island, Tasmania[b]	T300	I
Neoaplectana species N	Coonabarabran, N.S.W.[b]	N37	I
Steinernema kraussei	Z. Mracek, C.S.A.V., Czechoslovakia	SK2	I
		SK3/1	I
		SK3/2	M
		SK6	I
		SK8	I
		SK9	I
		SK10	I
		ST1	MS[c]
		ST2	MS
Undescribed Steinernematid Q1	Mirani, Queensland	Q1/1	I
		Q1/2	M

[a] I - surface sterilised infectives; M - monoxenic *in vitro* culture of nematode and symbiont; G - *G. mellonella* larva infected with the nematode. [b] Isolated from soil by the method of Bedding and Akhurst (1975). [c] MS - monoxenic culture supplied by Z. Mracek.

agar (NA) slants with polyether polyurethane sponge impregnated with a brei of pork kidney, bovine fat and water (Bedding, 1981).

Most nematode strains were cultured at 23°C; nematodes isolated from northern Queensland and the Northern Territory were cultured at 28° and *S. kraussei* at 18°.

Axenic cultures of *N. bibionis* T231, *N. feltiae* Agriotos, *N. glaseri* and the undescribed species M and N were established by the method of Poinar & Thomas (1966). The nematodes were cultured on raw, sterile rat kidney on NA slants. The same methods were used, and modified, in attempts to establish axenic cultures of *Heterorhabditis*.

The infective stage nematodes were stored in water aerated by an aquarium bubbler. Steinernematids were stored at 7° and heterorhabditids at 23°.

Bacteria

The sources of bacteria are listed in Tables 1 and 2.

Many of the symbiotic bacteria were isolated by macerating surface-sterilized infective stage nematodes. Approximately 50 nematodes were surface-sterilized by immersion in 0.1% (w/v) merthiolate for 2-3 hours; after being washed three times with sterile water, they were suspended in Dye's (1968) yeast-salts (YS) broth (Table 3) and macerated in a tissue homogeniser. Samples of the macerate were spread on NA or NBTA (Table 3).

Some of the bacteria were isolated from monoxenic *in vitro* cultures of the nematodes established by the method of Bedding (1981) or from the haemocoel of *G. mellonella* larvae 2-6 days after infection by the nematodes. Samples from *in vitro* culture or infected *G. mellonella* larvae were streaked onto NA or NBTA.

Table 2. Sources of microorganisms other than those associated with nematodes.

	Microorganism	Source
Bacteria :	*Bacillus cereus* subsp. *mycoides*	Isolated in the course of this study
	B. polymyxa	U.T.M.C. M14
	B. subtilis	U.T.M.C. M10
	B. thuringiensis	U.T.M.C. M13
	Cellulomonas sp.	U.T.M.C. M16
	Escherichia coli	A.T.C.C.[b] 25922
	Enterobacter cloacae	A.T.C.C. 13047
	Erwinia carotovora	U.T.M.C. P74
	Micrococcus luteus	U.T.M.C. M35
	Proteus vulgaris	A.T.C.C. 6380
		U.T.M.C. M40
	Pseudomonas fluorescens	U.T.M.C. M44
	Serratia sp.	U.T.M.C. M49
	Shigella sonnei	U.T.M.C. M50
	Staphylococcus aureus	U.T.M.C. M51
Yeasts :	*Candida albicans*	Australian Government
	C. krusei	Health Department
		Lab., Hobart
	Saccharomyces cerevesiae	U.T.M.C. M47

a U.T.M.C. - University of Tasmania Microbiology Collection.

b A.T.C.C. - American Type Culture Collection.

Table 3. Non-proprietary media used for growth of *Xenorhabdus*

Medium	Reference	Formulation
Medium C base	Dye (1968)	$NH_4H_2PO_4$, 0.5g; K_2HPO_4 0.5g; $MgSO_4 7H_2O$, 0.2g; NaCl, 0.5g; yeast extract, 1g; water, 1L; pH 6.8
Egg albumen agar	-	Trypticase soy agar + 0.11% (w/v) $CaCl_2$ + 1.25% (w/v) fresh egg albumen
GYCA	Dye (1968)	Glucose, 5g; yeast extract, 5g; $CaCO_3$, 40g; agar, 15g; water, 1L
NBTA	Akhurst (1980)	NA + 0.0025% (w/v) BTB[a] + 0.004% (w/v) TTC[b]
OY agar	Dye (1968)	$NH_4H_2PO_4$, 0.5g; K_2HPO_4, 0.5g; $M_9SO_4 7H_2$), 0.2g; NaCl, 5.0g; yeast extract, 0.8g, agar, 12.0g water, 1L.
Phenol red	Difco Laboratories (1953)	Beef extract, 1g; proteose peptone No. 3 (Difco), 10g; NaCl, 5g; water, 1L
Tergitol-7 agar	Difco Laboratories (1953)	Proteose peptone No. 3 (Difco), 5g; yeast extract, 3g; lactose, 10g; agar, 15g; tergitol-7, 0.1ml; BTB, 0.025g; TTC, 0.04g; water 1L
Tryptone agar	-	Tryptone, 11g; agar, 12g; water, 1L
TTCG	Lysenko and Weiser (1974)	Tergitol-7 agar + TTC without tergitol-7

[a] Bromothymol blue

[b] Triphenyltetrazolium chloride

Table 3 (continued)

Medium	Reference	Formulation
Medium X	Götz *et al.* (1981)	Bacteriological peptone, 4g; NaCl, 5; glucose, 4g; water 1L pH 7.4
YDC	Dye (1968)	Yeast extract, 10g; dextrose, 5g; $CaCO_3$, 20g; agar, 15g; water, 1L
YS agar	Dye (1968)	$NH_4H_2PO_4$, 0.5g; K_2HPO_4, 0.5g; $MgSO_4 7H_2O$, 0.2g; NaCl, 0.5g; agar, 12.0g; water, 1L
YS broth	Dye (1968)	YS agar without agar

Stock cultures of *Xenorhabdus* isolates were maintained at 12° on YS agar (Table 3). Cultures of other genera were maintained at 12° on NA. For long term storage, bacteria were deep-frozen (-18°) in 17% (w/v) glycerol/nutrient broth or freeze-dried in 5% (w/v) peptone 3% (w/v) sucrose at 10^{-3} Torr (about 0.1 Pa) and -70°, with subsequent storage at 4°.

Yeasts

The sources of yeasts are listed in Table 2. Yeasts were cultured on malt agar; stock cultures were incubated at 4°.

Insects

G. mellonella were cultured on a medium of Farex (Glaxo Australia) (50 g), glycerine (22 g), honey (23 g) and yeast (5 g).

Comparison of Methods for Viable Counts of Xenorhabdus

To compare spread plate and pour plate methods as means of estimating the number of viable *Xenorhabdus* cells, samples of serially diluted 24 hour YS broth cultures of *Xenorhabdus* isolate A24 were spread on NA or pipetted into sterile petri dishes to which NA at 45° was then added. Colonies were counted after incubation at 28° for 24, 48 and 72 hours.

Comparison of Media for the Growth of Xenorhabdus

NA, NA with yeast extract (Poinar & Thomas 1967), medium X (Table 3) and YS agar were compared as growth media for *Xenorhabdus*. Samples of serially diluted 24 hour YS broth cultures of *Xenorhabdus* isolate A24 were spread on the various media. Colonies were counted at a magnification of x10 after 24 hour incubation at 28°.

Incorporation of bromothymol blue into agar media facilitated the identification of *Xenorhabdus* spp. Three agar media containing bromothymol blue were compared as growth media for *Xenorhabdus*: tergitol-7 agar with triphenyltetrazolium chloride (TTC), Lysenko & Weiser's (1974) TTCG, and NBTA. Samples of a serially diluted 24 hour YS broth culture of *Xenorhabdus* isolate TN6 were spread on the various media. Colonies were classed according to colour and counted after 4 days at 28°.

Diluents

Sterile Ringer's solution (Cruickshank *et al.* 1970) was tested for its suitability as a diluent for *Xenorhabdus* spp. YS broth cultures (24 hour) of *Xenorhabdus* isolates were serially diluted with sterile Ringer's solution prior to spreading appropriate dilutions on YS agar immediately and 4.5 hours later. Colonies were counted at a magnification of x10 after 24 hour incubation at 28°.

TAXONOMY OF BACTERIAL SYMBIONTS OF THE STEINERNEMATIDAE AND HETERORHABDITIDAE

Bacterial Isolates

The bacteria used were those listed in Table 1 except B/2, C/2, D/2 and Q393/2.

Methods Used for the Characterisation of Isolates

Tests were conducted at 28° except those requiring shaking, which were conducted at 25°. In general, test media were inoculated from 1-6 day-old YDC agar (Table 3) cultures which produced a considerably higher

number of cells than NA or YS agar. For tests in which growth was examined, the medium was inoculated with a loopful of an aqueous suspension of cells from YDC agar cultures.

Average cell size was estimated by measurement of 50 cells in wet mounts of 24 hour YS broth cultures. Motility was assessed by examination of hanging drops of 24 hour YS broth cultures and the flagella position was determined by transmission electron microscopy after negatively staining cells from 24 hour YS broth cultures. Air dried films of 24 hour YS broth cultures were stained for the Gram reaction which was checked by treatment of a heavy suspension of cells from YS broth culture with 10% (w/v) sodium dodecyl sulphate (Hayes *et al.* 1978).

Colony and cultural characteristics were studied on NA, tergitol-7 agar with TTC, MacConkey agar (Difco), Simmons citrate agar (Difco), and triple sugar iron agar (Difco). Pigmentation of colonies was recorded after growth on NA and YDC agar.

Catalase activity was tested by flooding 24 hour NA cultures with 10% (v/v) hydrogen peroxide and also by placing a loopful of cells from a 24 hour NA culture into a drop of 10% (v/v) hydrogen peroxide on a glass slide. These methods were repeated with cultures on Dye's (1968) GYCA (Table 3). The following tests were conducted as described by Dye (1968): oxidation-fermentation, oxidase, hydrolysis of starch and of aesculin, methyl red, nitrate reduction, urease, KCN tolerance, maximum temperature for growth, reducing substances from sucrose, growth-factor requirements, and utilization of organic acids (using the OY medium). Gluconate utilization was also tested by the method of Shaw & Clarke

(1955). Acetoin production in shake-cultures was tested in Dye's (1968) acetoin medium and in Difco MR-VP medium after 2 and 5 day incubation as described by Dye (1968). Samples from shaken cultures in Dye's (1968) indole medium were tested for production of indole at 2 and 5 days with Kovac's reagent and also with Ehrlich's reagent after the addition of xylene. Casein hydrolysis was tested on Dye's (1968) OY agar containing 10% (v/v) skim milk.

Tyrosinase and chitinase tests were those used by Khan & Brooks (1977). Lecithinase and lipase were tested on YSA containing 5% (v/v) fresh egg yolk emulsion (20% (w/w) egg yolk in distilled water) and on tryptone agar (Table 3) containing 5% (v/v) fresh egg yolk emulsion. Lipase activity was also assessed on Sierra's (1957) medium with Tween 80 concentration reduced to 0.2% (v/v) to allow more vigorous growth. Gelatin hydrolysis was tested in nutrient gelatin and protease activity on Loeffler's blood serum and on egg albumen agar (Table 3).

Moeller's (1955) tests for arginine dihydrolase and lysine, ornithine and glutamic acid decarboxylases were used. The chromatographic methods of Stewart (1963) and McMeekin *et al.* (1973) were used as a check on the results of the glutamic acid decarboxylase test; *P. vulgaris* was used as a positive control.

DNase activity was tested as described in the Difco Laboratories (1962), cytochrome oxidase by Schaeffer's (1961) method, and peroxidase by Anderson's (1930). The test for phosphatase was conducted on phenolphthalein phosphate agar as described by Cowan & Steel (1974). Cultures on Difco phenylalanine agar were tested with freshly prepared reagents (Difco Supplementary Literature 1962) at 2, 5 and 7 days to assess phenylalanine deaminase activity.

Production of acid from carbon sources was assessed in 1% (w/v) peptone water containing brom cresol purple and 1% (w/v) carbon source, except aesculin which was 0.1% (w/v). Aesculin, inulin, and salicin were tyndallised in the medium; all other carbon sources were filter sterilised and added to the cooled, autoclaved basal medium. Bromothymol blue (0.002% [w/v]) and phenol red (0.0018% [w/v]) were tested for use as more sensitive indicators of acid production than brom cresol purple. YS broth, Dye's (1968) medium C base and phenol red broth base (using brom cresol purple, bromothymol blue or phenol red as indicator) were tested as basal media. Media were inoculated from NA, YDC agar and YS broth cultures to determine whether the medium from which inoculum was obtained affected the result. Tests for acid production were made in 10 ml broth in 15 ml McCartney bottles and in 20 ml test tubes fitted with aluminium caps to determine whether the container affected the result.

Bioluminescence was determined by examining 48 hour NA cultures for 10 minutes in total darkness.

The bouyant density of seven isolates was determined jointly by Dr. G. Skyring and Dr. E. Dennis, CSIRO, Canberra by ultracentrifugation with *Micrococcus luteus* as the standard. The guanine plus cytosine (G + C) content of the DNA was calculated from the formula of Schildkraut *et al.* (1962).

Antibacterial activity was tested with *B. cereus* subsp. *mycoides* by a variation of the method of Poinar, Hess & Thomas (1980).

Pathogenicity was tested by injecting approx. 10^3 cells (total count) of each isolate (from 24 hour YS broth culture) into the

haemocoel of final instar *Galleria* larvae; 20 larvae were injected with each isolate and a further 20 larvae were injected with sterile Ringer's solution as controls. Isolates were considered pathogenic if more than 10 died within 3 days. Non-pathogenic isolates were retested at dosages up to 10^6 cells/larva.

Taxonomic relationships between the isolates were examined by numerical analysis using the MACINF and GCOM packages of the Taxon Library, Edition P3 (CSIRO Division of Computing Research, 1982). Most of the data were processed in binary form; data on pigmentation and maximum temperature for growth were processed as disordered multi-state variables.

COLONY DIMORPHISM IN XENORHABDUS SPP.

Bacteria

Most of the investigation into colony dimorphism in *Xenorhabdus* was conducted with isolates A24 and A25 from *N. feltiae* Agriotos. However, bacteria isolated from other strains, species and genera of nematode were used to assess the general occurrence of features displayed by A24 and A25.

Differentiation of Colony Forms

Both forms of several isolates were subjected to the tests described above for the study of the taxonomy of bacteria associated with steinernematids and heterorhabditids. Production of antimicrobial compounds was tested with 17 species (25 strains) of bacteria.

The sensitivity of both forms of the bacterial symbionts of *N. feltiae* Agriotos, *N. bibionis* T335, *N. glaseri*, *Heterorhabditis* T280

and the Polish strain of *Heterorhabditis* was tested on Isosensitest agar (Oxoid) with Oxoid multidiscs U4 and 30-12L. The width of inhibition zones was measured after 24 hours incubation.

Differentiation of the forms of *Xenorhabdus* in the presence of various dyes was tested on nutrient agar (with or without 0.004% [w/v] TTC) containing one of the following: brom cresol purple, phenol red, methylene blue, alcian blue (all at a concentration of 0.0025% [w/v]); crystal violet (0.001% [w/v]); neutral red (0.003% [w/v]). The agar media were inoculated with either form of bacterial symbiont from each of the nematode species and examined after incubation for 3 days.

The pathogenicity of the two forms of the *N. feltiae* Agriotos symbiont was compared by estimation of the LD_{50} following intrahaemocoelic injection of *G. mellonella* larvae. Bacteria were grown for 24 hours in shaken YS broth cultures prior to estimation of cell concentrations by use of a counting slide. Each culture was then serially diluted with sterile Ringer's solution to produce seven concentrations ranging from approximately 0.05 to 50 cells μl^{-1}. Aliquots (10 µl) of each concentration were injected into 20 *G. mellonella* larvae using a 10µl syringe with 0.5 mm diameter cannula. Another 20 larvae were injected with sterile Ringer's solution. The larvae were incubated at 22° on dry filter paper for 3 days. LD_{50} was determined by probit analysis (Bliss, 1938). The LD_{50} values, transformed to logarithms, were compared by t-test.

Effect of the Bacterial Form on Nematode Reproduction

The two forms of the *N. feltiae* Agriotos symbiont were cultured separately in YS broth shaken at 25° for 24 hours and diluted with

sterile Ringer's solution to ca. 10^5 cells ml^{-1}. *G. mellonella* larvae (30) were injected with ca. 10^3 cells of one or other form of the symbiont and, 24 hours later, with 10 axenic infective stage *N. feltiae* Agriotos, thus avoiding the possible effect of live insect host on nematode growth. The *G. mellonella* larvae were incubated at 22°. After 5 days, and again after 8 days, five *G. mellonella* larvae were dissected and the length of nematode females measured at a magnification x10 using an eyepiece micrometer. When nematode reproduction was completed, the remaining 20 *G. mellonella* cadavers were dissected in water and the number of nematodes estimated from replicate samples taken from an agitated suspension. The foreguts of infective juveniles were examined by the method of Poinar (1966) to determine the proportion of infective juveniles containing bacteria. Bacteria were isolated from the nematodes by maceration, as described previously, to determine the form present.

The same method was used to determine the effect of symbiont form in the size and reproduction of *H. heliothidis* (using infective juveniles from monoxenic culture) and *N. bibionis*.

The effect of the form of symbiont on *N. glaseri*, *S. kraussei*, *Neoaplectana* sp. M and the undescribed steinernematid Q1 was assessed by injecting 20 *G. mellonella* larvae with 10 infective juveniles and ca. 10^4 cells of one or other form of the bacterial symbiont. The larvae were incubated at 23° on white traps (White 1929) and the numbers of infective juveniles emerging from the cadavers were estimated as described above.

The effect of the symbiont form on nematode reproduction was also tested in the absence of other microorganisms. Polyether polyurethane foam coated with a brei of pork kidney, beef fat and water (Bedding 1981) in 500 ml flasks was inoculated with one or other form of bacterial symbiont. The flasks were incubated at 23° for 2 days and then inoculated from monoxenic cultures of *H. heliothidis/X. luminescens* or *N. feltiae/X. nematophilus*. *H. heliothidis* flasks were harvested at 14 or 25 days, *N. feltiae* flasks at 15 days; the number of infective juveniles in each was estimated by counting samples as described above.

Stability of the Forms of Xenorhabdus

The stability of the bacterial forms against change to the alternative form was examined under a variety of conditions. Monoxenic cultures of nematodes and their bacterial symbionts on artificial media and cultures of either form of symbiont in peptone water and/or in YS broth, both shaken and stationary, were tested for a change of form over at least 30 days by streaking on NA or NBTA. Cultures of either form of the symbiont of *N. feltiae* Agriotos in YS broth were serially diluted with sterile Ringer's solution and spread onto NBTA. The numbers of colonies of either form were counted after incubation for 3 days. Pure cultures of either form were also incubated aerobically or anaerobically on NA. These cultures were subcultured three times at intervals of 3 days, with samples being streaked at each time onto NBTA and incubated aerobically.

The stability of the forms *in vivo* was also tested by sampling *G. mellonella* larvae infected by *N. feltiae* while buried in sand or injected with either form of the symbiont, with or without infective

juveniles. Each *G. mellonella* cadaver was dipped in ethanol, ignited and plunged into sterile Ringer's solution; the haemocoel was exposed and irrigated with the Ringer's solution. The liquid was serially diluted and spread on NBTA. Infective juveniles, harvested from injected and from naturally infected *G. mellonella* larvae were stored in water for 1-90 days and then macerated as described previously to determine the form of the symbiont. This method was also used for *N. bibionis* and *Neoaplectana* sp. M and their respective bacterial symbionts.

Deep-freezing and freeze-drying were tested as methods of storing pure cultures of the forms over long periods. In the former instance a loopful of bacteria from a 24 hour YS agar culture was dispersed in 5 ml nutrient broth containing 17% (v/v) glycerol and immediately deep-frozen at -18°. After 2, 6, 12, 52 and 65 weeks, cultures were rapidly thawed in a 56° water bath and subcultured onto NBTA. After incubation at 28° for 3 days, the plates were examined to determine the viability and stability of the forms.

Bacterial cultures to be freeze-dried were washed from 48 hour YS agar cultures with 5% (w/v) peptone/3% (w/v) sucrose and freeze-dried in a Dynavac FD16 high vacuum freeze-drying unit at 10^{-3} torr (ca. 0.1 Pa) and -70°; the ampoules were sealed under vacuum and stored at 4°. At various intervals up to 2 years, an ampoule of either form was opened, the contents reconstituted with sterile water and streaked onto NBTA. Viability and stability of the bacterial forms were determined after incubation for 3 days.

Attempted Isolation of Bacteriophage from Xenorhabdus

Both forms of the symbiont of *N. feltiae* Agriotos were tested for the presence of bacteriophage. A 24 hour, shaken YS broth culture of either form of the symbiont was diluted with YS broth until faintly turbid and then shaken for a further 3 hours. The exponential phase culture was pipetted into petri dishes to a depth of ca. 0.8 mm and exposed to UV radiation at 55 cm from a 15 w germicidal lamp for 0, 30, 60 or 300 seconds. Treated cultures were shaken in screw-cap bottles for 3 hours in the dark and then filter sterilised. The filtrates were added to YS broth (1:3) in screw-cap bottles. Two bottles of each filtrate/YS broth and two of untreated YS broth were inoculated with a loopful of 24 hour YS broth culture of the form being tested; another two bottles of each were similarly inoculated with the other form and one bottle of each filtrate/YS broth was kept uninoculated as a control. After incubation at 28° for 24 hours each bottle was sampled by streaking a loopful of broth onto NBTA. After incubation for 3 days the NBTA plates were examined to determine the stability of the forms.

The filtrates were also examined for lytic phage on agar overlay plates as follows: for each treatment, 0.2 ml of filtrate was pipetted onto two NA plates; 0.5 ml of a 24 hour YS broth culture of one form of the bacterial symbiont was added to one plate and 0.5 ml of a similar culture of the alternative form added to the other. The overlay was comprised of 0.7% purified agar (Oxoid) prepared with Ringer's solution. Plates were examined for plaque formation after 24 hours incubation at 28°.

Attempted Demonstration of Plasmids from Xenorhabdus

Both forms of the symbiont of *N. feltiae* Agriotos were examined for the presence of plasmid DNA by the methods for small scale, crude plasmid preparation and gel electrophoresis described by Hirsch *et al.* (1980).

Both forms of the symbiont of *N. feltiae* Agriotos were treated with mutagens in an effort to cure them of possible plasmids. YS broth and YS broth containing 50 μg ml^{-1} (w/v) sodium dodecyl sulphate, 50 μg ml^{-1} (w/v) acridine orange or 600 μg ml^{-1} (w/v) ethidium bromide were inoculated with 0.1 ml of a 24 hour YS broth culture. The cultures were shaken for 24 hours and subcultured (0.1 ml) into 5 ml of the corresponding medium. The new cultures were similarly subcultured after 24 hours. A sample was taken from each culture 24 hours after inoculation and streaked onto NBTA. After incubation for 3 days the NBTA plates were examined for a change of form of the bacteria.

In another attempt to cure the symbiont of *N. feltiae* Agriotos of possible plasmids, YS broth cultures of either form of the symbiont were incubated at 34°; they were subcultured twice at 24 hour intervals. Samples taken from each culture after 24 hours were streaked onto NBTA; the NBTA plates were incubated at 28° for 3 days and examined for a change of form of the bacteria.

ANTIMICROBIAL ACTIVITY OF *XENORHABDUS* SPP.

Media

Except where otherwise specified *Xenorhabdus* strains were cultured in YS broth; other strains of bacteria were cultured in nutrient broth

(Difco). Yeast strains were grown on malt agar (Oxoid) and suspended in nutrient broth prior to use. All incubation was at 28°C.

Sensitivity Tests

NA plates were spot inoculated from 24 hour broth cultures of *Xenorhabdus* strains and incubated for 3 days. The *Xenorhabdus* were then killed by exposing the plates to chloroform for 2 hours. After the plates had been left 1 hour to allow evaporation of the chloroform, 1.0 ml of a 24 hour culture of the test organism was added to each plate. Sterile soft agar (nutrient broth 5.0 g, Bacto-agar 7.0 g, distilled water 1L) at 45° was poured into each plate which was then agitated to evenly disperse the inoculum. Widths of inhibition zones were noted after 24 hours.

Potency of Antibacterial Activity in Xenorhabdus spp.

Xenorhabdus strains were inoculated into 5 ml YS broth, nutrient broth or 1% peptone water in 15 ml screw cap bottles. The bottles were incubated either shaken or stationary, for up to 10 days. Cultures of various ages were sterilised by shaking with 0.1 ml chloroform for 1-2 hours. The cultures were then serially diluted and 2 ml aliquots, of each dilution pipetted into two petri dishes. Sterile Isosensitest agar (Oxoid) at 45° was added to each petri dish which was then agitated and allowed to set. The plates were then streaked with a loopful of a faintly turbid suspension of *Micrococcus luteus* from a 24 hour nutrient broth culture and examined for growth of *M. luteus* after 24 hours.

Effects of Aeration, Heat and Dialysis on Antibacterial Activity

The effect of aeration on production of antibacterial activity was examined using *X. nematophilus* A24 cultured in peptone water. Cultures

initiated in petri dishes containing a shallow layer of medium and in 15 ml screw cap bottles containing 5 ml or 10 ml of medium were tested daily for 5 days as described above. Nutrient agar plates were spot inoculated from 24 hour broth cultures of each of several strains of *X. nematophilus* and *X. luminescens* and incubated anaerobically for 6 days. Antibacterial activity was tested as described previously using *Micrococcus luteus* and *B. cereus* subsp. *mycoides* as test organisms.

The effect of heat on antibacterial activity was assessed with 5 day nutrient broth cultures of *X. nematophilus* A24 (5 ml in 15 ml screw cap bottles). Cultures were either incubated at 60°C for 10 minutes, autoclaved at 121°C for 15 minutes or unheated. All cultures were then treated with 0.1 ml chloroform and tested in Isosensitest agar as described above.

The effect of dialysis was also assessed with 5 day nutrient broth cultures of *X. nematophilus* A24. The cultures were sterilised with chloroform; two cultures were kept as control and another two dialysed in running water overnight to remove molecules with molecular weight less than 14 000. The control cultures and dialysates were tested as previously described.

Defective Phage

The method of Poinar, Hess & Thomas (1980) was used to examine *X. nematophilus* A24 and A25 (primary and secondary form, respectively) for phage-like particles.

SPECIFICITY OF THE NEMATODE/BACTERIUM ASSOCIATION

Monoxenic Cultures

Monoxenic cultures were established in duplicate, on polyurethane foam coated with Bedding's (1981) medium on NA slants. The medium was inoculated with axenic nematodes and 1-4 day YS broth cultures of bacteria. Control cultures without bacteria were inoculated with axenic nematodes. Cultures were incubated at 23° and subcultured when the medium was exhausted (2-3 weeks).

Cultures were rated as successful if nematode reproduction continued after 3 serial subcultures. When a combination of nematode and bacterium did not culture successfully, at least one repeat attempt at culture was made.

Estimation of the Proportion of Infective Juveniles Containing Bacteria

Approximately 50 infective juveniles from each successful nematode/bacterium culture were examined microscopically for bacteria after extrusion of the foregut and staining with crystal violet or safranin (Poinar 1966). For each nematode/bacterium combination, the proportion of infective juveniles containing bacteria was estimated by calculating the mean of percentage values obtained from each culture after transformation by arcsin $\sqrt{}$ proportion.

The viability of bacteria in the infectives was determined by inoculating NA plates with a homogenate of 100 surface sterilised infective juveniles. The NA plates were examined after incubation at 28° for 3 days.

The results obtained with the microscopic method were compared with those obtained by sampling *Galleria* haemolymph in which individual,

surface sterilised infective juveniles had exsheathed (Poinar 1966). Data were analysed by X^2 test.

Variation in Proportion of Infective Stage N. glaseri Containing Bacteria

The proportion of infective stage *N. glaseri* containing bacteria was also determined with infective juveniles emerging from various species of insect and infective juveniles harvested from monoxenic *in vitro* culture flasks (Bedding 1981).

G. mellonella larvae were obtained from laboratory cultures described previously. *Heliothis punctiger* and *Tenebrio molitor* were cultured in the laboratory by the methods of Shorey & Hale (1965) and Helms & Rawn (1971) respectively. *Adoryphorous couloni* and *Lepidiota frenchi* larvae were collected from the field.

Insect larvae were buried in moist sand containing infective stage *N. glaseri*. Infected larvae were removed after several days and placed individually in White traps (White 1929) from which emerging infective juveniles were collected. At least 50 infective juveniles from each insect were examined microscopically for the presence of bacteria in the intestine.

Infective stage *N. glaseri* emerging from a *T. molitor* larva were grouped into long (> 1.2 mm) and short (< 1.0 mm) classes. Samples of each class were examined for the presence of bacteria in the foregut both microscopically and by isolation in haemolymph. The conspecificity of the two classes was tested by the cross-breeding method (Appendix I). Groups of 10 infective juveniles from either class were injected with 10 µl of a 10-fold dilution of a 24 hour YS broth culture of the

bacterial symbiont into *G. mellonella* larvae. Infective juveniles emerging from the *G. mellonella* larvae were measured and examined for the presence of bacteria in the intestine.

PATHOGENICITY OF *XENORHABDUS* SPP.

The concentrations of cells in 20-24 hour YS broth cultures of *Xenorhabdus* isolates and of the *Flavobacterium* isolate ST1 were estimated by means of a counting slide. The cultures were then serially diluted with sterile Ringer's solution to obtain the required dosages.

For each bacterial isolate tested, a range of 7-9 dosages and a sterile Ringer's solution control were injected into the haemocoel of final instar *G. mellonella* larvae (20 larvae/dosage). Injections were made with a 10 µl syringe fitted with a 0.5 mm diameter cannula. To minimise microbial contamination from the surface of the insect larvae, the syringe was rinsed three times with 95% ethanol and then three times with sterile Ringer's solution following the injection of each group of five larvae. Injected larvae were incubated at 23° on dry filter paper and the number of dead recorded after 3 days. Where possible, data were analysed using the Probit analysis method of Finney (1971).

Effect of Nematodes on Pathogenicity of Xenorhabdus

Groups of 20 *Galleria* larvae were injected with sterile Ringer's solution or 1 or 2 axenic *N. glaseri* infectives as well as with 0, 115, 380 or 1150 cells of *Xenorhabdus* isolate G/1 (total count). Injected larvae were incubated at 23° on dry filter paper and the number of dead recorded after 3 days. Data were analysed by analysis of variance by assuming that the binomial response (dead or living) can be treated as a normally distributed continuous variable.

INCIDENCE OF BACTERIA OTHER THAN *XENORHABDUS* IN NEMATODE-INFECTED INSECTS

G. mellonella larvae were placed individually into 100 ml specimen jars and covered with moist sand; approximately 100 infective stage nematodes were then pipetted onto the surface of the sand. After incubation at 23° for 24 hours, the *G. mellonella* larvae were removed and stored on dry filter paper at 23°. At appropriate intervals two larvae were sampled aseptically as follows: each larvae was dipped in 99% ethanol, ignited and immersed in 5 ml sterile Ringer's solution. The insect cuticle was opened with sterile forceps and the body cavity irrigated 10 times with the Ringer's solution (care being taken to avoid rupturing the intestine). The resulting liquid was then serially diluted and spread on NA plates. After incubation at 28° for 2-3 days, the plates were examined and the numbers of *Xenorhabdus* and other colonies counted.

G. mellonella larvae were similarly infected in soil with *N. feltiae* Agriotos infective juveniles containing either primary or secondary form symbiont. Four of the larvae infected with *N. feltiae*/primary form symbiont and four infected with *N. feltiae*/secondary form symbiont were sampled for bacteria after 3 days and another four from each group after 6 days. The sampling method was similar to that described above but with the addition that samples were spread on NBTA, *Salmonella-Shigella* agar and desoxycholate citrate agar as well as NA. Colonies were identified as *Xenorhabdus* or "other" and counted after incubation at 28° for 3 days.

RESULTS

Establishment and Maintenance of Axenic Cultures of Nematodes

Axenic cultures of *Neoaplectana* spp. were established and maintained on raw, sterile rat kidney. Axenic cultures of *Heterorhabditis* spp. could not be established because the axenic nematodes were unable to utilize raw, sterile rat kidney or liver.

Comparison of Media and Methods for the Cultivation of Xenorhabdus

The pour plate method gave a much lower estimate of the number of *Xenorhabdus* cells because the bacterium grew very poorly in the agar.

The growth of *Xenorhabdus* isolate A24 was better in YS agar than on any other medium tested (Table 4). However, the colony morphology of *Xenorhabdus* spp. was not as distinctive on YS agar as on NA based media. NBTA was more satisfactory for the growth of *Xenorhabdus* than either of the other media containing bromothymol blue (Table 5). The estimation of the proportions of red and blue colonies did not differ significantly between the three media.

Xenorhabdus spp. did not suffer significant loss of viability during storage in sterile Ringer's solution for 4.5 hours (Table 6).

TAXONOMY OF BACTERIAL SYMBIONTS OF THE STEINERNEMATIDAE AND HETERORHABDITIDAE

Colony Morphology

Each of the bacterial symbionts of steinernematid and heterorhabditid nematodes may produce two forms of colony. The form normally isolated from infective juveniles is designated the primary form. This form is unstable under many conditions (detailed later in the section "Colony Dimorphism in *Xenorhabdus* spp.") and produces the secondary form.

Table 4. Comparison of agar media for the culture of *Xenorhabdus*[a]

Medium	Experiment No.	Estimate of cells YS agar / Estimate of No. cells using other medium	P
NA	1	3.6	<0.05
	2	3.5	n.s.
NA + 0.2% yeast extract	3	6.0	<0.05
0.5%		4.0	<0.05
1.0%		6.0	<0.05
1.5%		9.4	<0.05
X[b]	4	1.8	<0.05
	5	1.2	n.s.

[a] Samples of 24 hour YS broth culture of *Xenorhabdus* isolate A24 were serially diluted in sterile Ringer's solution; 0.1 ml aliquots were spread on 3 plates of YS agar and 3 of the other medium (10 plates when medium X was tested). Colonies were counted after incubation at 28° for 24 hours. Data analysed by t-test.

[b] Bacteriological peptone, 4g; NaCl, 5g; glucose, 4g; pH 7.4; Götz *et al.* (1981).

Table 5. Comparison of agar media containing bromothymol blue for growth of *Xenorhabdus*[a].

Medium	Estimated number of *Xenorhabdus* (cells of ml^{-1} of orignial culture)	S.E.	% Colonies Red	% Colonies Blue
Tergitol-7 + TTC[b]	$1.3.10^4$	$0.4.10^4$	55	45
TTCG[c]	$8.3.10^4$	$0.5.10^4$	45	55
NBTA[d]	$6.1.10^5$	$1.0.10^5$	50	50

a Samples of 24 hour YS broth culture of *Xenorhabdus* isolate TN6 were serially diluted in sterile Ringer's solution; 0.1 ml aliquots were spread on 2 plates of each medium. Colonies were counted according to their colour after 4 days incubation at 28°.

b Proteose peptone No. 3 (Difco), 5.0g; Bacto-yeast extract, 3.0g; Bacto-lactose, 10.0g; Bacto-agar, 15.0g; tergitol-7, 0.1 ml; Bacto-bromthymol blue 0.025g; water 1L; triphenyltetrazolium chloride (TTC), 0.04g.

c Tergitol-7 agar + TTC minus tergitol-7

d Nutrient agar, 23g; bromothymol blue, 0.025g; water, 1L; TTC, 0.04g.

Table 6. Suitability of sterile Ringer's solution as a diluent for *Xenorhabdus* spp.[a]

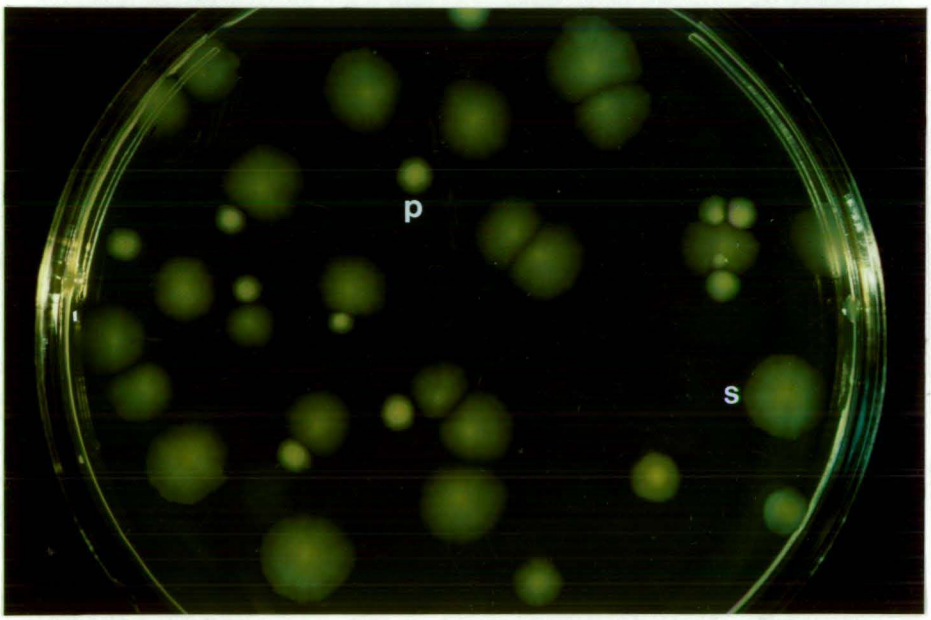
Xenorhabdus isolate	No. Colonies (S.E.) t = 0	No. Colonies (S.E.) t = 4.5 hours	P
C/1	210 (58)	183 (37)	n.s.
G/6	34 (39)	59 (19)	n.s.
A24	95 (32)	119 (52)	n.s.

[a] 24 hour YS broth cultures were serially diluted with sterile Ringer's solution; 0.1 ml aliquots were spread on 20 YS agar plates immediately or after 4.5 hours at room temperature (ca. 20°). Colonies were counted at a magnification of x10 after 24 hours incubation at 28°. Data were analysed by t-test.

On NA the primary form colonies of bacteria symbiotic with *Heterorhabditis* spp. were mucoid, convex, circular with a slightly irregular margin and 3 mm diameter at 4 days, except T280/1 which was slightly flattened and 4 mm diameter. The pigmentation of the colonies of some isolates varied with time, all were yellow at 2 days with some progressing through orange to red or through a light brown to pink. The secondary form colonies of these bacteria were much flatter and wider (4.5 mm diameter at 4 days) than the primary form, with a more irregular margin and were not mucoid (Fig. 1). Two of the secondary form isolates were further differentiated; T280/2 formed rough colonies and HP/2 formed yellow colonies while T280/1 formed smooth and HP/1 formed orange colonies. Colonies of the primary form on tergitol-7 agar + TTC were green with an orange centre and surrounded by a clear zone diffused with pigment. Secondary form colonies, except 280/2, did not absorb bromothymol blue (BTB) from T7 + TTC and produced red colonies.

Colonies formed on NA by the primary form of bacteria symbiotic with *Neoaplectana* spp., *S. kraussei* and the undescribed steinernematid Q1 were convex, circular with slightly irregular margin, 1.5-2.0 mm diameter after 4 days and slightly granular; pigmentation varied with nematode species (Table 8). The secondary form colonies of these bacteria were similar to the primary but somewhat flatter, wider (2.5-3.5 mm diameter) and more lightly pigmented. On tergitol-7 agar + TTC, the primary form colonies of bacteria symbiotic with *N. bibionis* and *S. kraussei* were green-blue and those of *N. feltiae* and the steinernematid Q1 were blue; all were surrounded by a decolorized zone after 3-5 days. The primary form colonies of bacteria isolated from

Fig. 1 Primary (p) and secondary (s) form colonies of *X. luminescens* on NA showing differences in pigmentation and colony size.



Neoaplectana species M did not absorb BTB as strongly as the above and were reddish-blue with a partially decolorized zone. Secondary form colonies of bacteria symbiotic with the Steinernematidae did not absorb BTB and were red on tergitol-7 agar + TTC (the bacteria associated with *Neoaplectana* sp. N and the Tasmanian populations of *Neoaplectana* sp. M occurred only in the secondary form). Neither form of the bacterium isolated from infective stage *N. glaseri* absorbed BTB and both produced red colonies on tergitol-7 agar + TTC. However, the form isolated from infective juveniles absorbed neutral red and crystal violet from MacConkey agar to produce red colonies while the other form did not.

Isolate ST/1, from the monoxenic culture of *S. kraussei* and its "symbiont" provided by Dr. Z. Mráček, formed yellow, "fried egg" colonies on NA with diameter of 3-4 mm at 4 days. ST/2 colonies were similarly pigmented but were circular, convex with a smooth margin and were 1 mm in diameter. On tergitol-7 agar + TTC, ST/1 produced orange colonies that were convex, circular and surrounded by a wide, flat apron; the colonies of ST/2, except for the apron, were similar.

Characterisation of Bacterial Isolates

Assessment of acid production from carbohydrates was complicated by the production of acid from the basal medium by the symbionts of the Steinernematidae and Heterorhabditidae. Varying the basal medium (YS broth, Dye's 1968 medium C, phenol red broth base), the medium used to produce inoculum, or the container used (bottle or test tube) had no effect on the production of acid from the basal medium. Phenol red (pH range 6.8-8.4) and bromothymol blue (pH 6.0-7.6) were of no use as indicators because they changed colour in response to acid produced from

the basal medium by the bacteria; brom cresol purple (pH 5.2-6.8) also showed some colour change in response to this acid production. Consequently, results were assessed against a blank control (basal medium only, inoculated with bacterium) with brom cresol purple as indicator; a positive result was recorded only when a distinct yellow response was obtained.

The two chromatographic methods used to test for glutamic acid decarboxylase confirmed the negative results obtained with Moeller's (1955) method.

The bacteria isolated from infective stage *S. kraussei* required a larger inoculum than those of other groups for growth in both media; they also grew very poorly on Dye's (1968) OY medium.

The results of tests are summarised in Tables 7-9; details are presented in Appendix II.

Anaylsis of Taxanomic Data

Analysis of data produced six major groups (Fig. 2). However, the GCOM analysis showed that 56.4% of the information used to separate groups E & F and 30.55% of that used to separate groups C & D derived from three characters that occurred in the primary form only of bacterial symbionts of the Steinernematidae. When these characters (antibacterial activity, BTB absorbtion and lecithinase) were deleted and the data re-analysed, only two of the six major groups formed (G & H) had the same composition as groups in the original analysis (Fig. 3).

Table 7. Characteristics common to all isolates[a].

Characteristic	Result[b]	Characteristic	Result
Gram stain	-	DNase	+
Cell morphology	Rod	Potato starch hydrolysis	-
Acetoin	-	Soluble starch hydrolysis	-
Methyl red	-	Chitinase	-
Lysine decarboxylase	-	KCN tolerance	+
Ornithine decarboxylase	-	Protease - Loeffler's medium	-
Glutamic acid decarboxylase	-	egg albumen agar	-
Arginine dihydrolase	-	Tyrosinase	-
Production of gas from		Lipase - egg yolk agar	+
adonitol	-	MacConkey agar (growth on)	+
aesculin	-	Simmons nitrate agar	n
arabinose	-	Utilisation (in Shaw & Clark's medium) of gluconate	-
galactose	-	Utilisation (in OY medium) of	
inulin	-	acetate	+
lactose	-	benzoate	-
mannitol	-	citrate	+
melibiose	-	fumarate	+
β-methyl-glucoside	-	malate	+
raffinose	-	oxalate	-
rhamnose	-	succinate	+
sorbose	-	tartrate	-

[a] See Appendix II for list of isolates.

[b] Symbols: +, positive; -, negative; n, no growth.

Table 8. Characteristics not common to all isolates[1].

CHARACTERISTIC	BACTERIAL SYMBIONTS OF							
	Xenorhabdus spp.	*X. bovienii*	*X. poinarii*	*H. glaseri*	*Neoaplectana* spp. M,N	*S. kraussei*	none[2]	Undescribed Steinernematid 01
Mean cell length (μ)	5.7	5.8	6.0	4.5	5.2	5.5	2.7	5.4
Mean cell width (μ)	1.0	1.0	1.1	1.2	1.1	1.1	0.5	1.0
Motility	+	+	+	+	+	+	-	+
Flagella[3]	Pt	Pt	Pt	Pt	Pt	Pt	none	Pt
Pigmentation[4]	y,br,o, r,bu,p	y	bu	br	br	y	y	c
Catalase	+	-	-	-	-	-	+	-
Oxidase	-	-	-	-	-	-	+	-
Cytochrome oxidase	-	-	-	-	-	-	+	-
Peroxidase	-	-	-	-	-	-	+	-
Bioluminescence	+	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	-	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	-	+
Gelatin liquefaction	+	+	+	+	+	+	-	+
Casein hydrolysis	+	15+,1-	+	+	6+,3-	+w	-	+
Reducing compounds from sucrose	-	-	-	-	-	-	+	-
Urease	+	-	-	-	-	-	+	-
Aesculin hydrolysis	+	-	-	-	+	-	-	+
Lipase (Tween 80)	+	+	3+,5-	+	+	6+w,1±	+	+
Lecithinase	12+,1±	14+,2-	6+,2-	-	3+,6-	6+w,1-	-	1+,1-
Phenylalanine deaminase	6+,7-	1±,15-	2+,6-	-	-	-	+	-
Indole	3+,10-	-	-	-	-	-	-	-
Phosphatase	+	1+,15-	-	-	+	-	+	1±,1-
Antibacterial activity	12+,1-	15+,1-	6+,2-	-	3+,6-	6+,1-	-	1+,1-
Pathogenicity	+	+	+	-	+	+	-	+
Absorbtion of BTB	12+,1-	15+,1-	6+,2-	-	3+,6-	6+,1-	-	1+,1-
MacConkey - red colonies	11+,2-	-	-	1+,1-	-	-	-	-
Mucoid colonies	+	-	-	-	-	-	-	-
Triple sugar iron agar[4]	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	0/0/n	+/-/n
Growth at 34°	8+,5-	-	+	+	+	-	+	+
36°	2+,11-	-	-	+	6+,2-	-	-	+
38°	2+,11-	-	-	+	3+,6-	-	-	-
40°	-	-	-	+	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	-	+
gluconate	12+,1-	15+,1-	4+,4-	+	+	+	+	-
propionate	12+,1-	12+,4-	+	+	+	+	+	+
Acid from dextrin	+w	+w	+w	+w	+w	+w	±	+w
fructose	+	+	+	+	+	+	-	+
glucose	+	+	+	+	+	+	+w	+
glycerol	-	2+w,9±,5-	-	+w	1+w,8-	-	-	+w
inositol	9±,4-	11±,5-	4±,4-	1±,1-	6±,3-	-	-	-
maltose	1+,12+w	+w	+w	1+,1+w	7+,2+w	+w	-	1+,1+w
mannose	12+,1+w	+	+	1+,1+w	+	6+,1+w	±	+
melezitose	10+w,3±	15+w,1±	7+w,1±	1+w,1±	+w	5+w,2±	±	+w
α-methyl-glucoside	-	3±,13-	-	-	-	-	-	-
ribose	12+w,1-	+w	+w	+w	4+w,3±,2-	3+w,1±,3-	+w	+w
saccharose	-	3±,13-	-	-	-	-	-	±
salicin	2±,11-	-	-	-	3+w,6-	-	-	-
sorbitol	1±,12-	3±,13-	-	-	-	-	-	-
sucrose	1±,12-	2±,14-	3±,5-	-	-	-	±	±
trehalose	11+w,2±	+w	+w	+w	1+,8+w	+w	±	+w
xylose	1±,12-	5±,11-	3±,5-	-	1±,8-	-	-	-

[1] Symbols: +, all positive; +w, weakly positive; ±, doubtful; -, all negative.

[2] Isolates ST1, ST2 - from Mrácek's monoxenic culture (see Table 1).

[3] Pt, peritrichous.

[4] y, yellow; br, brown; o, orange; r, red; bu, buff; p, pink.

[5] +/-/n: alkaline slope, acid butt, no H_2S; 0/0/n: no change in slope or butt, no H_2S.

Table 9. DNA base ratio of some *Xenorhabdus* isolates.

Bacterial isolate	Associated nematode species	Bouyant density (g cm^{-3})	G + C (mol %)
*Micrococcus luteus**	-	1.731	72.4
T310	*Heterorhabditis* sp.	1.707	48.0
T231	*N. bibionis*	1.706	46.9
A24	*N. feltiae*	1.707	48.0
A25	*N. feltiae*	1.707	48.0
G/2	*N. glaseri*	1.708	49.0
N37	*Neoaplectana* sp. N	1.709	50.0
T171	*Neoaplectana* sp. M	1.708	49.0

* Included as standard. Skyring and Jones (1972) measured the bouyant density of this strain against the reference strain *E. coli* K12 which has a bouyant density of 1.7100 g cm^{-3}.

Fig. 2 Dendrogram derived from full data set by MACINF (Taxon Library, P3 edition, CSIRO Division of Computing Research, 1982). Groups A-F are discussed in the text.

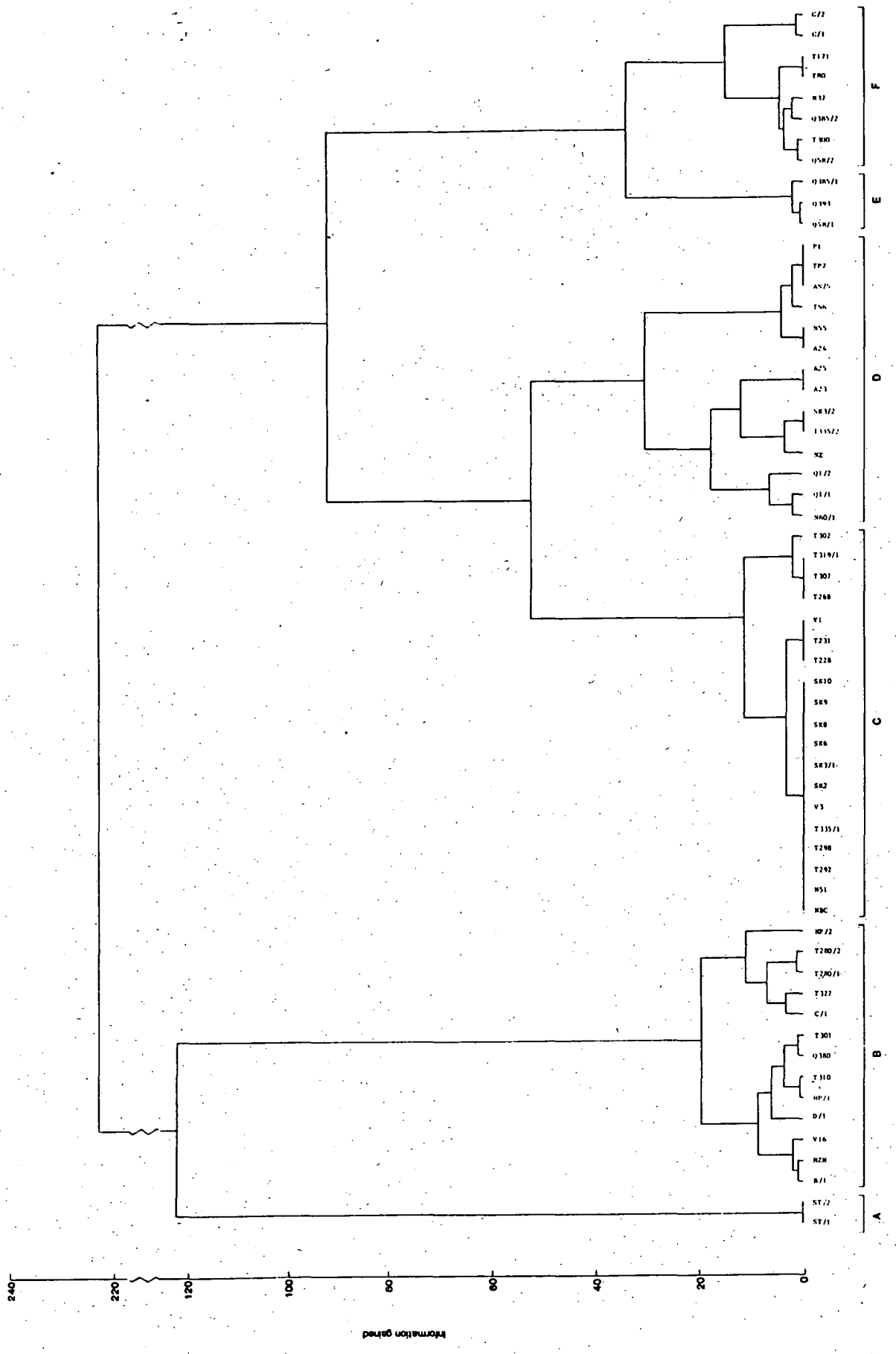
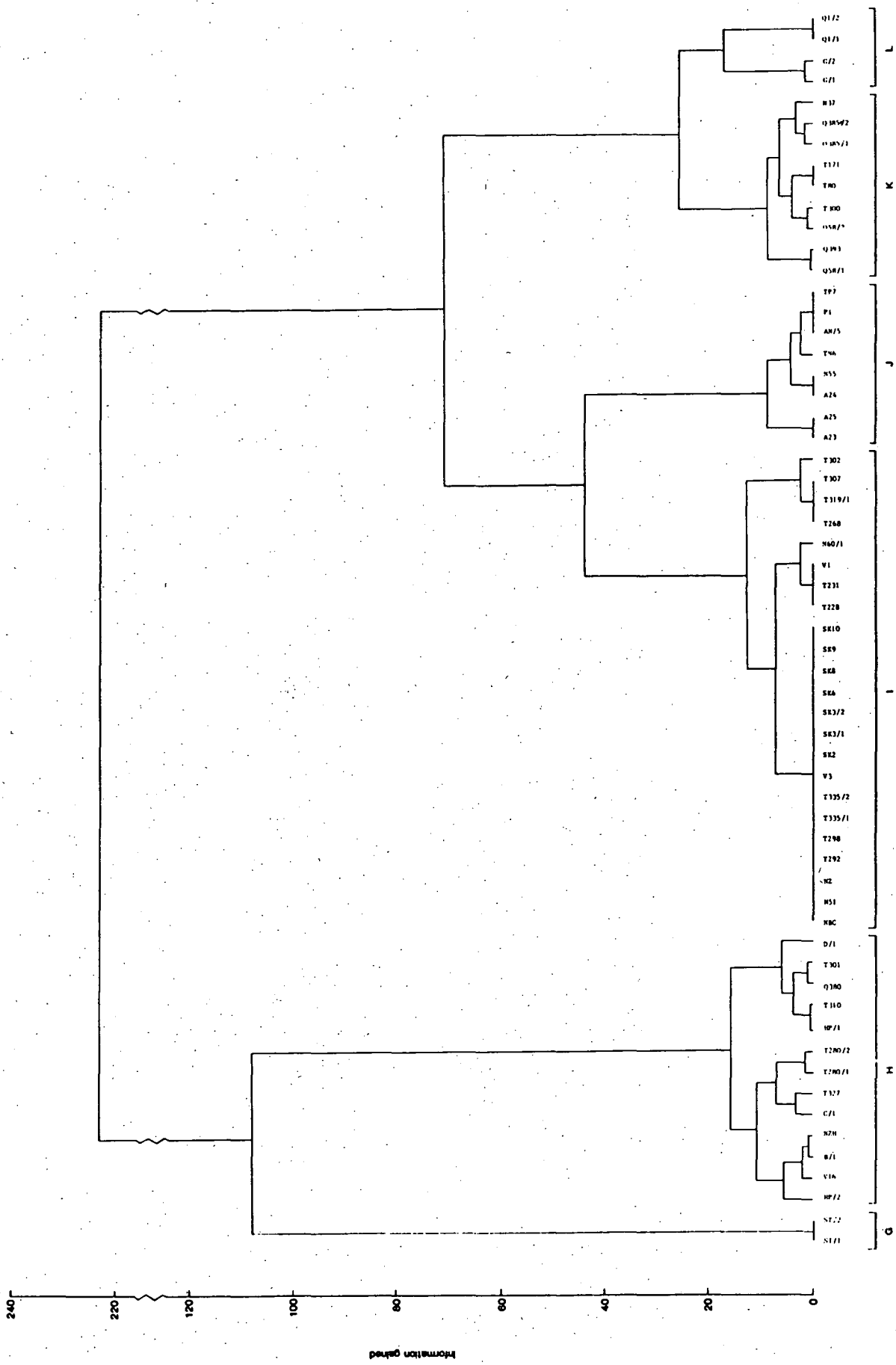

C/2
C/1
T171
T80
N32
Q385/2
T100
Q58/2
Q385/1
Q393
Q58/1
F1
TP7
A57
T56
S55
A24
A25
A23
SR3/2
T335/2
SZ
Q1/2
Q1/1
S60/1
T302
T319/1
T307
T268
V1
T231
T228
SR10
SR9
SR8
SR6
SR3/1
SR2
V3
T335/1
T298
T292
N51
RBC
RP/2
T280/2
T280/1
T327
C/1
T301
Q380
T310
RP/1
D/1
V16
NZH
B/1
ST/2
ST/1
F
E
D
C
B
A
240
220
120
100
80
60
40
20
0
Information gained

Fig. 3 Dendrogram derived from data (not including data for antibacterial activity, BTB absorbtion and lecithinase) by MACINF (Taxon Library, P3 edition, CSIRO Division of Computing Research, 1982). Groups G-L are discussed in the text.


L
K
J
I
H
G
240
220
120
100
80
60
40
20
0
Information gained

COLONY DIMORPHISM IN *XENORHABDUS* SPP.

The bacterial symbionts of *H. bacteriophora* (1 strain), *H. heliothidis* (2), unidentified *Heterorhabditis* spp. (8), *N. bibionis* (15), *N. feltiae* (6), *N. glaseri* (1), *S. kraussei* (1) and the unidentified steinernematid Q1 (1) all produced two forms of colony on agar media. The symbionts of three strains of *Neoaplectana* sp. M also produced two colony forms. A further three strains of *Neoaplectana* sp. M and one strain of the closely related (Bedding pers. comm) *Neoaplectana* sp. N produced only one form.

In those nematode species whose symbiont occurred in two forms, infective stage nematodes normally contained only one of the forms, the primary form. The secondary form was isolated from monoxenic *in vitro* cultures inoculated with the primary form and occasionally from the cadavers of nematode-infected *G. mellonella* larvae.

Differentiation of the Colony Forms

Agar media containing brom cresol purple, bromothymol blue, methylene red, crystal violet or neutral red (but not phenol red) were useful for identifying the two forms. Most primary form *Xenorhabdus* colonies absorbed these dyes from the agar, becoming intensely coloured and decolorising the agar after 2-3 days (neither form of the symbiont of *N. glaseri* was able to absorb brom cresol purple or bromothymol blue). With the exception of *X. luminescens* T280/2, secondary form colonies were unable to absorb these dyes. Secondary form colonies did appear to be lightly coloured because of the colour of the agar on which they grew. Inclusion in media of TTC, which was reduced to formazan (red) by both forms facilitated their identification because secondary

form colonies were red while primary form colonies were the same colour as the dye (the dye absorbed by the colonies masked the red) (Fig. 4).

There was no difference in pathogenicity or antibiotic sensitivity between the two forms of any symbiont and few differences in their reactions to biochemical tests. In general, the primary form of bacteria symbiotic with steinernematid nematodes produced lecithinase while the secondary form did not. Isolates A23 and A25, secondary form of the symbiont of *N. feltiae* Agriotos, gave positive results for phenylalanine deaminase and lipase (Tween 80 test); isolate A24, the primary form did not.

The two forms differed in antimicrobial activity. The primary form of all symbionts, except that of *N. glaseri*, produced clear inhibition zones in lawns of various microorganisms; the secondary form, at best, caused a slight reduction in the density of the lawn (Fig. 6).

There was no significant difference in the pathogenicity of the primary and secondary form of the *N. feltiae* Agriotos symbiont (LD_{50} = 3 and 2 respectively.

Effect of the Bacterial Forms on Nematode Reproduction

When infective stage nematodes were injected onto *G. mellonella* larvae previously injected with the primary form of the symbiont, adult females grew larger and produced a greater number of infective juveniles than in larvae injected with the secondary form (Tables 10,11). The nematodes also matured more rapidly in the presence of the primary form symbiont emerging as infective juveniles several days before those from *G. mellonella* injected with the secondary form. In some instances *G. mellonella* larvae injected with the secondary form became heavily

Fig. 4 Primary (p) and secondary (s) form colonies of *X. nematophilus* NBTA showing absorbtion of bromothymol blue by primary form colonies only.

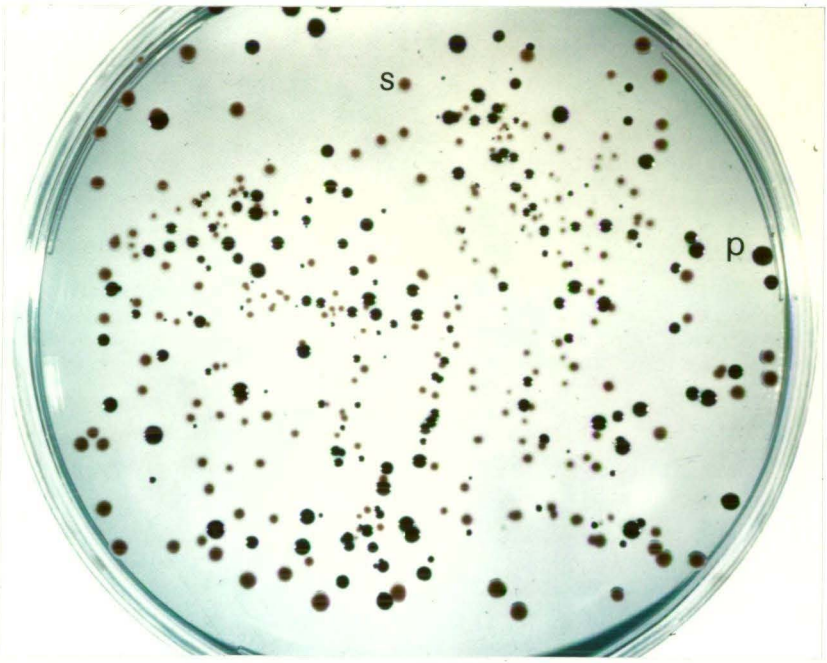


Table 10 Effect of form of *Xenorhabdus* on resultant growth of female nematodes following injection of infective juveniles into *G. mellonella* larvae[a].

Time after injection of nematodes (days)	Form of *Xenorhabdus*	*N. feltiae* length (mm)	P	*N. bibionis* length (mm)	P	*H. heliothidis* length (mm)	P
5	Primary	12.2	<0.001	4.5	<0.001	4.1	<0 .001
	Secondary	4.1		2.3		1.5	
8	Primary	11.7	<0.001	-[b]		not	
	Secondary	4.9		2.0		measured	

a 10 *G. mellonella* larvae were injected with primary or secondary form symbiont and, after 24 hours, with 10 infective juveniles. The larvae were incubated at 22°. After 5 days, nematodes were dissected from 5 larvae and the length of adult females (1st generation) measured. Data were analysed by analysis of variance.

b First generation females had been destroyed by emergence of their progeny (*endotrika matricida*).

Table 11. Effect of *Xenorhabdus* on yield of infectives from *G. mellonella* injected with infective stage nematodes and their bacterial symbionts.

Nematode	Estimated No. infectives produced per *G. mellonella* larva by:	
	Primary Form	Secondary Form
N. bibionis	26,100	3,600
N. feltiae	107,000	15,000
N. glaseri	12,400	10,500
	9,100	7,600
Neoaplectana sp. M	53,000	6,000
S. kraussei	9,500	0[c]
Undescribed steinernematid Q1	28,350	18,130
H. heliothidis	45,000[a]	22,000
	69,500[b]	0[c]

[a] New Zealand strain

[b] North Carolina strain

[c] Cadavers heavilty infected with fungus

contaminated with other microorganisms, particularly fungi, and the nematodes died within the cadaver either as infectives or at an earlier stage of development (see *S. kraussei*, *H. heliothidis*; Table 11).

Stability of the Forms of Xenorhabdus spp.

The primary form of all *Xenorhabdus* isolates was unstable, converting to the secondary form. However, the degree of instability was highly variable within and between taxonomic groups of *Xenorhabdus* (Fig. 3). The primary forms of bacteria isolated from strains of *Neoaplectana* sp. M were the least stable producing the secondary form after 2-3 days in culture; in contrast, axenic cultures of the symbionts of *H. heliothidis* and *N. feltiae* DD136 rarely produced the secondary form. The symbionts of the Agriotos and Nelson strains of *N. feltiae* were much less stable than those of the DD136 and Pieridarum strains.

Pure cultures of the primary form of all isolates of *Xenorhabdus* could be maintained by freeze-drying, deep-freezing or by frequent subculture (some requiring subculture every 48 hours when incubated at 28°). When broth cultures (shaken or stationary) or agar cultures that had been incubated at 28° for more than 7 days were subcultured onto agar media, some of the resulting colonies were secondary form. However, conversion of a primary form culture to secondary form was never complete (Table 12) and it was often difficult to establish a pure culture of either form by selecting colonies from these subcultures. *In vitro* monoxenic cultures of nematodes and primary form *Xenorhabdus* invariably produced the secondary form, though sometimes not for several weeks and after several subcultures. The primary form of most *Xenorhabdus* isolates was stable in *G. mellonella* larvae, whether or not nematodes were present. However, the primary forms of the symbionts of

Table 12. Instability of primary and secondary forms of *X. nematophilus* symbiont of *N. feltiae* Agriotos in YS broth at 28°.[a]

Age of culture (d)	Primary Form Mean % conversion	s.e.	Secondary Form Mean % conversion	s.e.
3	0	0	0	0
7	0	0	23.0	17.0
10	0.3	0.8	12.5	6.9
25	20.1	29.0	0.9	0

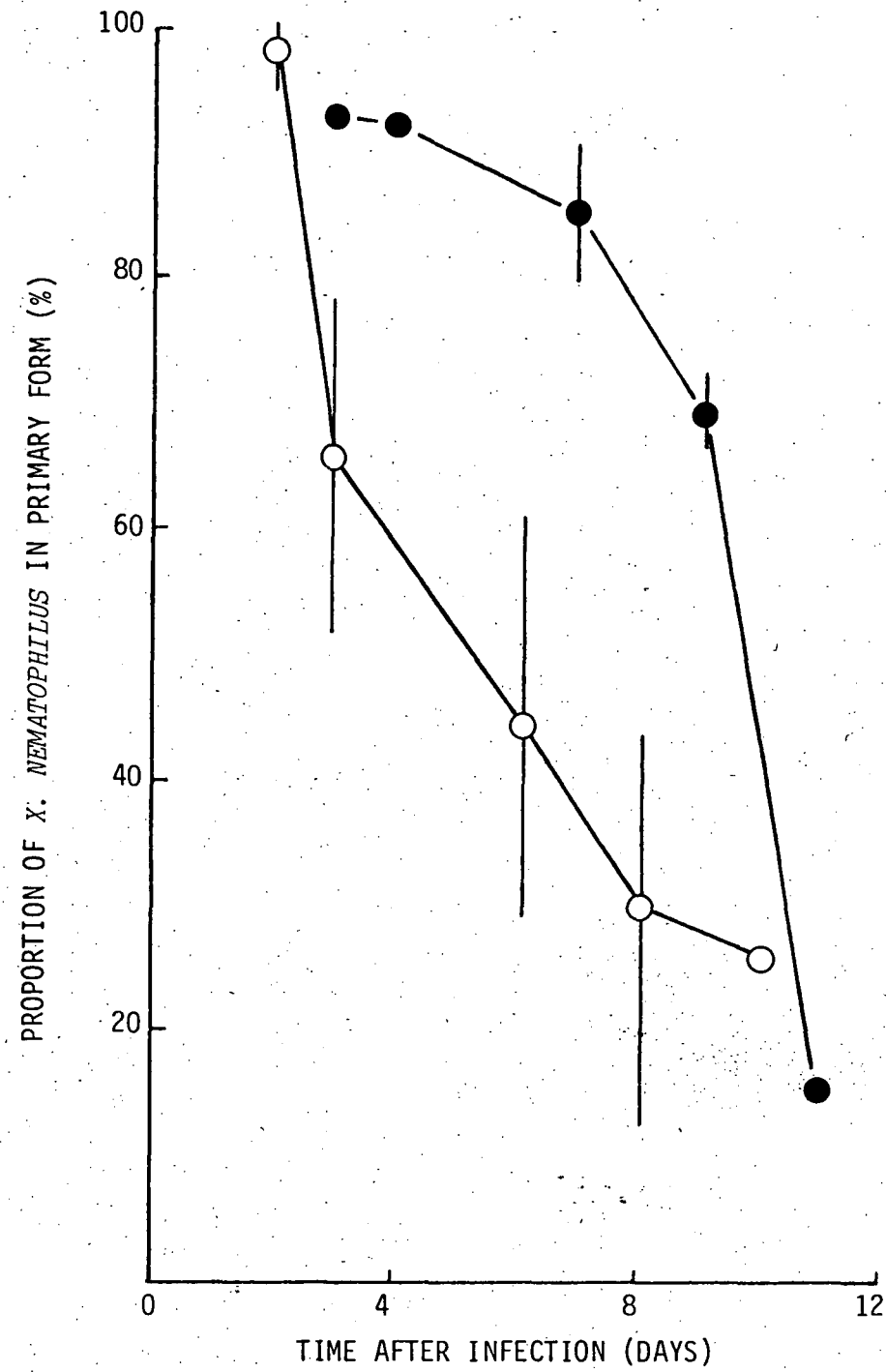
[a] These cultures of either form were incubated (not shaken) at 28°. At each sampling time samples of each culture were serially diluted in sterile Ringer's solution and spread on NBTA plates. Colonies were classified after incubation at 28° for 3-5 days.

nematodes were present. However, the primary forms of the symbionts of some strains of nematode were unstable in cadavers in the presence of nematodes (Fig. 5).

The secondary form of the *N. feltiae* symbiont was stable on aerobically incubated agar media, in monoxenic *in vitro* culture with nematodes and in *G. mellonella*, with or without nematodes. However subculture from (unshaken) broth media demonstrated a partial reversion to primary form (Table 12) that occurred after maximum cell number had been reached. Subculture of secondary form cultures grown anaerobically on NA also produced both primary and secondary form colonies. The secondary forms of the symbionts of *N. bibionis* and of *S. kraussei* were also unstable in broth culture; those of *N. glaseri*, *Neoaplectana* species M & N, *H. heliothidis*, and the undescribed steinernematid Q1 did not produce the primary form.

Infective *N. feltiae* were able to carry either form of their symbiont. Infectives obtained from *in vitro* cultures initiated with primary form symbiont or *G. mellonella* injected with primary form carried within their intestines only the primary form of the symbiont; those obtained from *in vitro* culture or *G. mellonella* where only the secondary form was present carried the secondary form. In *G. mellonella* injected with *N. feltiae* and both forms of its symbiont only 4% of the symbiont was in the primary form (cf. Fig. 5). When infectives produced from these *G. mellonella* were surface sterilised and placed individually in sterile *G. mellonella* haemolymph to exsheath and release their symbiont, a small proportion (3/20) were found to contain both forms.

Fig. 5 Proportion of *X. nematophilus* in the primary form in *Galleria* larvae incubated at 22° after natural infection by Agriotos and Nelson strains of *N. feltiae*.



Two larvae were sampled at each time; serial dilutions of dissected larvae were spread on NA, incubated at 28°, and assessed after 4-6 days. Experiments were terminated when the recovery of *X. nematophilus* from the larvae was too low to give a realistic estimate of the proportion of *X. nematophilus* in the primary form. For both Agriotos and Nelson varieties, the estimate of proportion at the last sampling was based on the result from one cadaver only. Mean and S.E. were calculated on arcsin √percentage transformed data. Agriotos strain ○; Nelson strain ●. Vertical lines represent S.E.

Attempts to Determine the Cause of the Change of Form of X. nematophilus

There was no evidence for the involvement of bacteriophage in mediating the change from one form to the other. Neither filtrates of UV-irradiated nor of untreated broth cultures of either form of the *N. feltiae* Agriotos symbiont produced plaques in lawns of either form of the symbiont on agar plates, nor did they induce a change of form in broth cultures of either form of this bacterium.

Incubation at near lethal temperature or in the presence of acridine orange or ethidium bromide failed to cause a change of either form of *X. nematophilus*. In YS broth containing sodium dodecyl sulphate there was some conversion of secondary form to primary; the conversion occurred earlier and to a greater degree in standing rather than shaken cultures.

No evidence of plasmid DNA in either form of *X. nematophilus* was found by extraction and electrophoresis using the methods described.

ANTIMICROBIAL ACTIVITY OF *XENORHABDUS* SPP.

With the exception of the symbiont of *N. glaseri*, all primary form *Xenorhabdus* inhibited the growth of a variety of microorganisms (Table 13). Colonies of secondary form or of anaerobically grown *Xenorhabdus* spp. did not prevent the growth of any microorganism tested although in some cases growth of the test organism was less dense around such colonies than elsewhere on the plate (Fig. 6).

The size of the inhibition zone varied with the test organism, its initial density and the thickness of the agar. However, *X. luminescens*, *X. nematophilus* A24 and *Xenorhabdus* sp. Q1 consistently produced larger zones of inhibition with most organisms than did *X. nematophilus* Q58

Table 13. Spectrum of antibiotic activity of the primary form of various *Xenorhabdus* isolates.

Indicator species	Inhibition by Xenorhabdus[a]								
	A24	T319/1	SK3/1	Q58/1	G/1	Q1/1	B1/1	C/1	HP/1
Cellulomonas sp.	+	+	+	+	-	+	+	+	+
M. luteus	+	+	+	+	-	+	+	+	+
S. aureus	+	+	+	+	-	+	+	+	+
B. cereus subsp. *mycoides*	+	+	+	+	-	+	+	+	+
B. polymyxa	+	+	+	+	-	+	+	+	+
B. subtilis	+	+	+	+	-	+	+	+	+
B. thuringiensis	+	+	+	+	-	+	+	+	+
E. coli	+	+	+	-	-	+	+	+	-
Sh. sonnei	+	+	+	-	-	+	-	-	+
Ent. cloacae	+	-	-	+	-	+	-	+	-
Serratia sp.	+	+	+	+	-	+	+	+	+
Pr. vulgaris	-	+	+	-	-	-	-	-	-
Erw. carotovora	+	+	+	+	-	+	-	+	-
X. luminescens NC/1	-	+	+	-	-	-	-	-	n
NC/2	-	+	+	-	-	-	-	-	n
X. nematophilus A24	-	+	+	+	-	+	+	+	+
A25	-	+	+	+	-	+	+	+	+
T319/1	-	-	-	-	-	-	+	+	+
T319/2	-	-	-	-	-	-	+	+	+
Q58/1	-	+	-	-	-	-	-	+	+
G/1	+	+	+	+	-	-	+	+	+
Xenorhabdus sp. SK/1	+	-	-	-	-	+	-	+	n
Xenorhabdus sp. Q1/1	-	-	-	-	-	-	-	+	+
Flavobacterium sp.	-	-	-	-	-	-	+	+	+
Ps. fluorescens	-	-	-	-	-	-	-	-	-
C. albicans	-	+	-	-	-	+	+	-	n
C. krusei	+	-	-	+	-	+	-	+	n
S. cerevesiae	+	+	+	+	-	+	-	+	n

[a] +, inhibition; -, no inhibition; n, not tested.

Fig. 6 Growth of *B. cereus* subsp. *mycoides* in an agar overlay on chloroform-killed colonies of *X. nematophilus* (right) and *X. luminescens* (left). Upper colonies : primary form; lower colonies : secondary form.

Table 14. Monoxenic *in vitro* culture of various combinations of *Neoaplectana* spp. and *Xenorhabdus* spp.[a]

Bacterium	Nematode species from which bacterium was isolated	Successful combinations				
		N. bibionis	*N. feltiae*	*N. glaseri*	*Neoaplectana* sp. M	*Neoaplectana* sp. N
None		-	-	-	-	-
X. nematophilus	*N. bibionis*	+(9)	-(4)	+/-(4)	-(3)	+(1)
	N. feltiae	+(4)	+(5)	+(2)	+(3)	+(1)
	N. glaseri	+(2)	+(2)	+(2)	+(2)	+(1)
	Neoaplectana sp. M	+(3)	+(2)	+(4)	+(5)	+(1)
	Neoaplectana sp. N	+(1)	+(1)	-(1)	+(1)	+(1)
	S. kraussei	+(1)	-(1)	-(1)	-(1)	n
X. luminescens	*Heterorhabditis*	-(8)	-(5)	-(5)	-(3)	-(1)
Xenorhabdus sp.	Undescribed steinernematid Q1	-(1)	-(1)	-(1)	-(1)	n
Flavobacterium sp.	-[c]	+(2)	-(2)	+(2)	+(1)	+(1)

[a] Axenic nematodes and bacteria were inoculated in various combinations onto Bedding's (1981) medium on NA slants. Nematode/bacterium combination was rated as successful if it could be maintained through 3 serial subcultures at 23°.

[b] Figures in brackets represent the number of bacterial isolates tested. Primary and secondary forms were counted as separate isolates.

[c] Isolated from *C. abietis* infected with *S. kraussei*.

n Not tested.

Fig. 7 Foregut of infective juveniles extruded and stained by Poinar's (1966) method. A. *N. feltiae* B. *N. bibionis* C. *N. glaseri* b. bacteria o. basal bulb of oesophagus (x 1000).

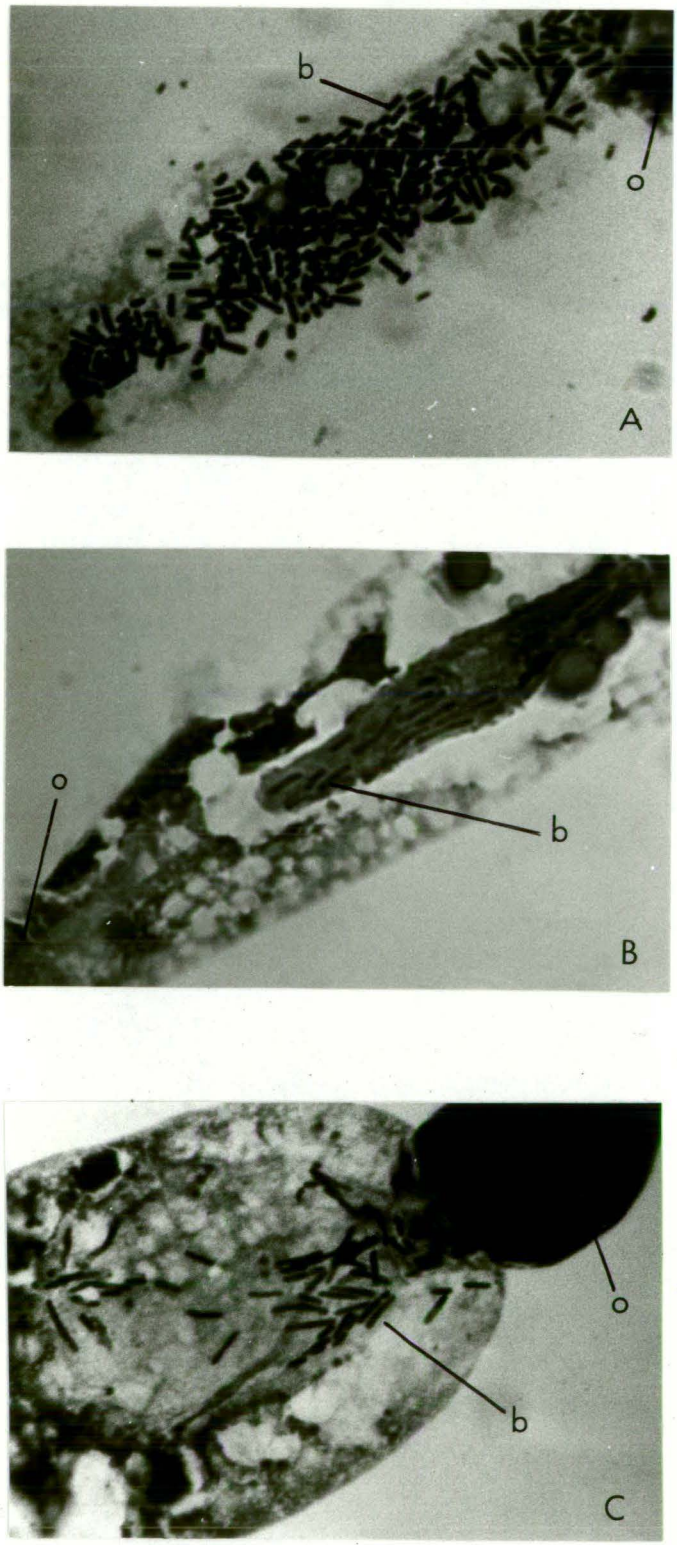
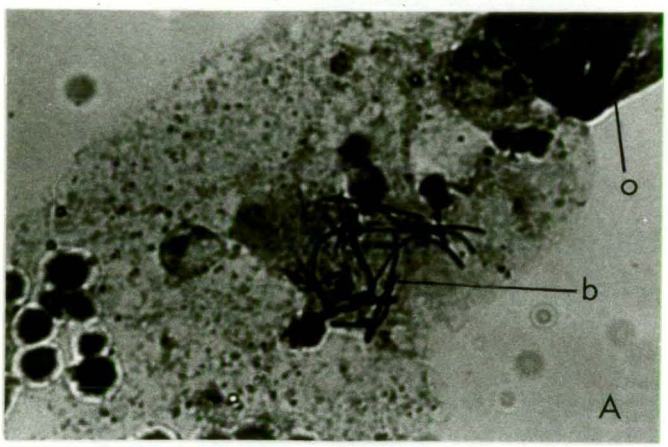


Fig. 8 Foregut of infective juveniles extruded and stained by Poinar's (1966) method. A. *Neoaplectana* sp. M B. *Neoaplectana* sp. N. b. bacteria o. oesophageal bulb of oesophagus (x 1000).



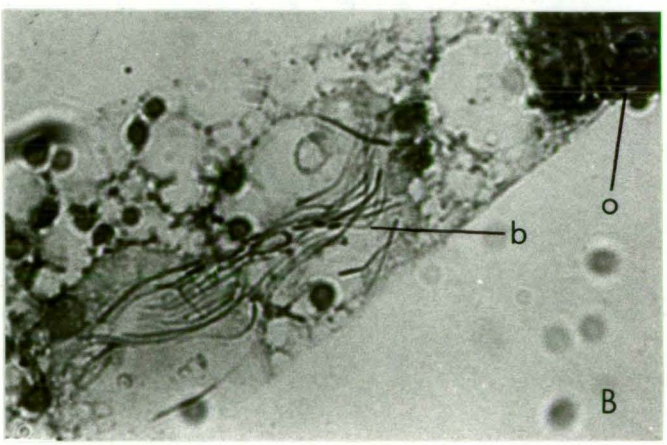


Table 15. Comparison of two methods for determining the proportion of infectives carrying bacteria with the intestine.

Nematode	Bacterium	Percentage of infectives carrying bacteria: Microscopic Method	Haemolymph Method	X^2	P
N. glaseri	Symbiont of *N. glaseri*	61	64	0.47	0.5
N. bibionis	Symbiont of *Neoaplectana* sp. N	12	10	0.09 ns	0.75
Neoaplectana sp. M	Symbiont of *Neoaplectana* sp. N	33	55	3.27 ns	0.05
		38	35	0.08 ns	0.75

which in turn produced larger zones than *X. nematophilus* T319 or *Xenorhabdus* sp. SK3/1.

Antimicrobial activity of broth cultures was always poor and could not be detected at dilutions greater than 50-fold. Antimicrobial activity of cultures in YS broth, nutrient broth or peptone-water was detected at low (10-fold) dilution in the petri dish cultures at 2 days and in screw cap bottle cultures with 5 ml broth at 3 days. At 5 days antimicrobial activity was detected in 50-fold dilutions of either culture though never in 10-fold dilutions of cultures in 10 ml broth in screw cap bottles.

Antimicrobial activity was not noticeably affected by heating either at 60° for 10 minutes or 121° for 15 minutes. No antimicrobial activity was detected after dialysis.

No particles resembling phage or parts of phage were detected.

SPECIFICITY OF THE NEMATODE/BACTERIUM ASSOCIATION

The results of attempts to culture various combinations of nematodes and bacteria are presented in Table 14. The nematodes reproduced most prolifically when cultured with the primary form of a bacterium isolated from any strain of that nematode species. In unsuccessful nematode/bacterium combinations, there were almost always too few nematodes produced initially to allow even one subculture.

The presence of bacteria in the intestine of infective juveniles was clearly detectable by the microscopic method (Figs. 7,8). There was no significant difference between estimates of the proportion of infectives containing bacteria obtained by the microscopic method and by isolation from haemolymph (Table 15).

Infectives of any *Neoaplectana* sp. were able to carry within their intestines the symbiont of any strain of the same nematode species. Some infectives of each *Neoaplectana* sp., except *N. feltiae*, were able to carry the symbiont of at least one other nematode species (Table 16).

Bacteria within Infective Stage N. glaseri

For *N. glaseri* infective juveniles from individual hosts or from individual culture flasks, the proportion containing bacteria varied considerably (Table 17). No insect species consistently produced a high proportion of infectives that contained bacteria.

The proportion of short (< 1.0 mm) *N. glaseri* infective juveniles containing the symbiont was highly significantly less than the proportion of long (>1.2 mm) infective juveniles ($\chi^2 = 8.04$, $P < 0.005$). However, the short infective juveniles interbred successfully with the long and when short infective juveniles were injected with their symbiont into *G. mellonella* larvae, they produced both classes of infective juvenile.

PATHOGENICITY OF *XENORHABDUS* SPP.

Injection of *G. mellonella* larvae with sterile Ringer's solution was rarely fatal; none of the larvae died in most of the control groups and in no group did more than two die. Deaths in the control groups were ignored in the calculation of LD_{50} because they occurred so infrequently and would have had little effect on the outcome.

Table 16. Transmission of *Xenorhabdus* within the intestine of infective stage *Neoaplectana*[a]

Bacterium	*N. bibionis*	*N. feltiae*	*N. glaseri*	*Neoaplectana* sp. M	*Neoaplectana* sp. N
Symbiont of *N. bibionis*	94[b] (2.3)	n	0	n	0
N. feltiae	0[c]	88[b] (0.7)	0	0	0
N. glaseri	0[c]	0	49[b] (1.4)	0	0
Neoaplectana sp. M	0[c]	0	7 (1.1)	40[b] (6.3)	7 (0.1)
Neoaplectana sp. N	10[b] (0.6)	0	n	35[b] (0.8)	75[b] (1.6)
S. kraussei	83[b] (1.0)	n	n	n	n
Flavobacterium sp.	0	n	0	0	0

[a] Infective stage juveniles from monoxenic *in vitro* cultures with their bacterial symbiont and those of other nematodes were examined microscopically for the presence of bacteria in the foregut. At least 50 infective juveniles from two or more cultures of each nematode/bacterium combination were examined. Percentages of nematodes containing bacteria from each culture were transformed by arcsin $\sqrt{P}$ for calculation of mean and S.E.

[b] Viable bacteria isolated from homogenized surface-sterilised infectives.

[c] Bacteria not isolated from homogenized surface-sterilised infectives.

n No infectives available.

Table 17. Effect of insect host on proportion of infective stage *N. glaseri* containing bacteria[a].

Source of infective stage *N. glaseri*	No. of individual sources	% infective stage *N. glaseri* containing bacteria	Range
G. mellonella	8	38.3	10-65
H. punctiger	4	24.0	15-31
A. couloni (3rd instar)	4	33.0	12-47
L. frenchi (2nd instar)	6	35.7	13-48
L. frenchi (3rd instar)	3	27.3	0-56
T. molitor	5	34.8	4-47
Monoxenic mass culture flasks	3	25.8	17-40

[a] Infective stage juveniles were harvested from individual insects infected with *N. glaseri* or individual flasks of *N. glaseri* culture. At least 50 nematodes from each source were examined microscopically for the presence of bacteria in the intestine. Means were calculated after transformation of percentages by arcsin $\sqrt{P}$.

Dosage mortality curves for *G. mellonella* injected intrahaemocoelically with bacteria are presented in Figs. 9-12. The LD_{50} values are given in Table 18.

Effect of Nematodes on Pathogenicity of Xenorhabdus

There was significantly ($P < 0.001$) greater mortality among *G. mellonella* injected with axenic *N. glaseri* and *Xenorhabdus* isolate G/1 than among those injected with either nematode or bacterium alone (Table 19, Appendix IV). Over the ranges tested, there was no significant difference between mortality resulting from the various dosages of nematodes or of bacteria and there was no significant interaction when at least one nematode was used in conjunction with more than one bacterium (Appendix IV).

INCIDENCE OF BACTERIA OTHER THAN *XENORHABDUS* IN NEMATODE-INFECTED INSECTS

The proportion of *Xenorhabdus* in the bacterial flora of nematode-infected *G. mellonella* larvae was very high (> 95%) between 2 and 4 days after infection and usually declined at some time thereafter (Figs. 13,14). However, the decline in the proportion of *Xenorhabdus* was not due to a large increase in the number of other bacteria but merely a decline in the number of *Xenorhabdus*.

The proportion of *Xenorhabdus* in *G. mellonella* larvae infected with *N. bibionis* was low (23%) 9 days after infection (Fig. 14). However, 9 days after infection most of the *N. bibionis* in the *G. mellonella* larvae were already in the infective stage.

Fig. 9 Dosage/mortality responses following intrahaemocoelic injection of final instar *G. mellonella* larvae with the symbionts of *N. bibionis* T231 and *N. feltiae* Agriotos (primary and secondary forms). Vertical lines represent S.E.

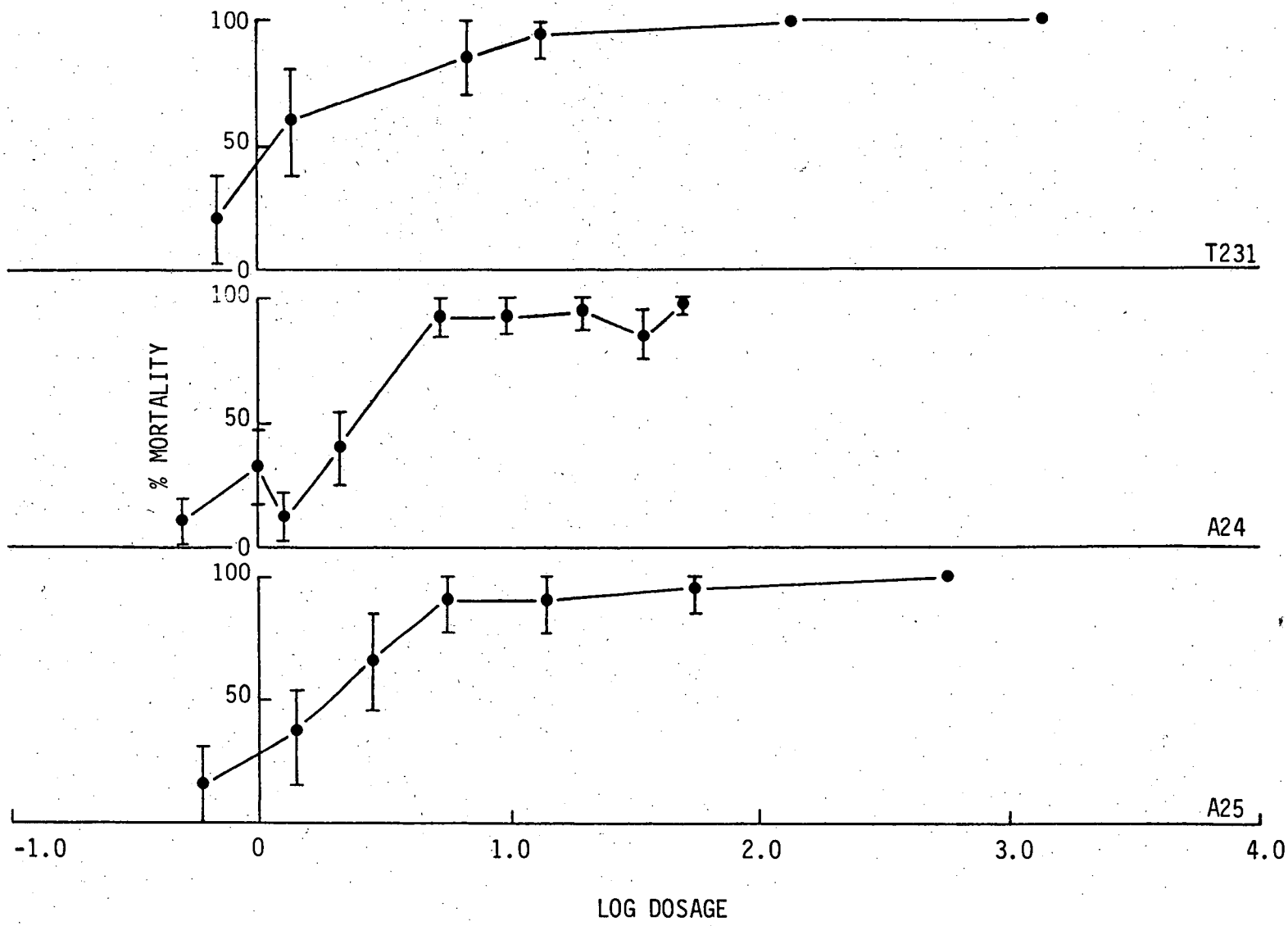

100
50
0
T231
100
50
0
% MORTALITY
A24
100
50
A25
-1.0
0
1.0
2.0
3.0
4.0
LOG DOSAGE

Fig. 10 Dosage/mortality responses following intrahaemocoelic injection of final instar *G. mellonella* larvae with the symbionts of *H. bacteriophora*, *Heterorhabditis* sp. T301, and *S. kraussei*. Vertical lines represent S.E.

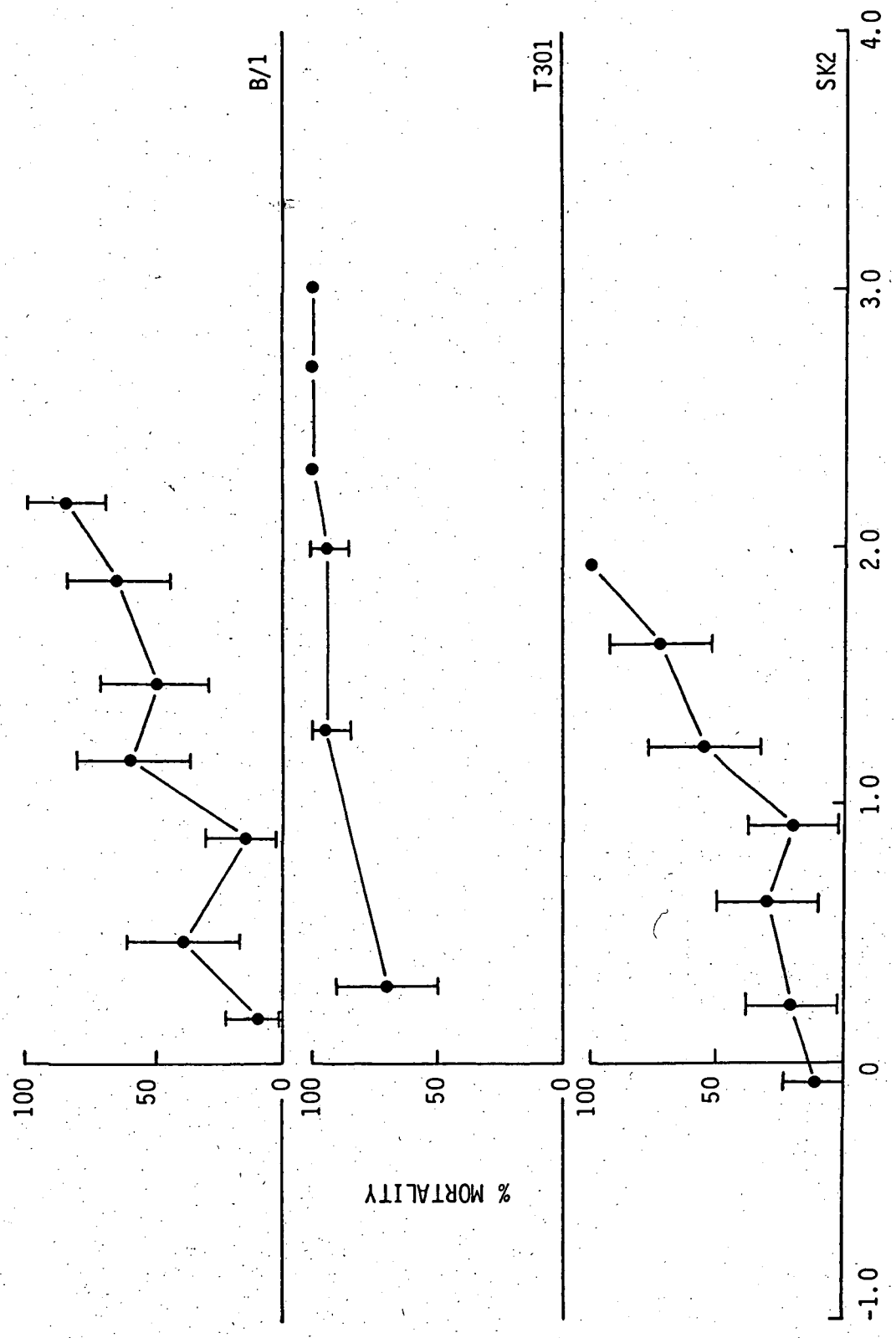

B/1
T301
SK2
100
50
0
% MORTALITY
-1.0
0
1.0
2.0
3.0
4.0
LOG DOSAGE

Fig. 11 Dosage/mortality responses following intrahaemocoelic injection of final instar *G. mellonella* larvae with the symbionts of the Q385 and T171 strains of *Neaoplectana* sp. M and *Neoaplectana* sp. N. Vertical lines represent S.E.

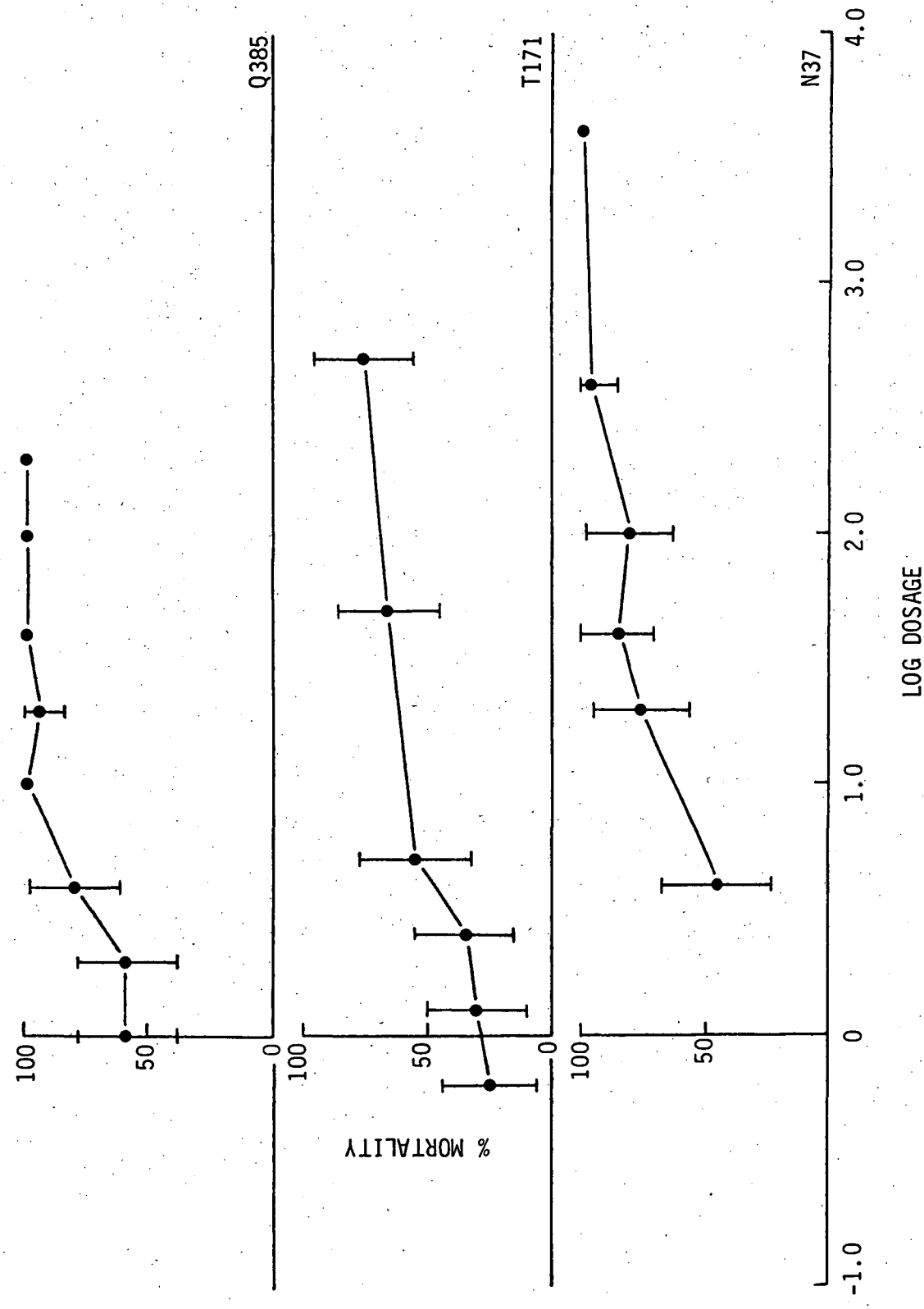

Q385
T171
N37
100
50
0
% MORTALITY
-1.0
0
1.0
2.0
3.0
4.0
LOG DOSAGE

Fig. 12 Dosage/mortality responses following intrahaemocoelic injection of final instar *G. mellonella* larvae with the *Flavobacterium* sp. ST1 and the symbionts of the steinernematid Q1 and of *N. glaseri*. Vertical lines represent S.E.

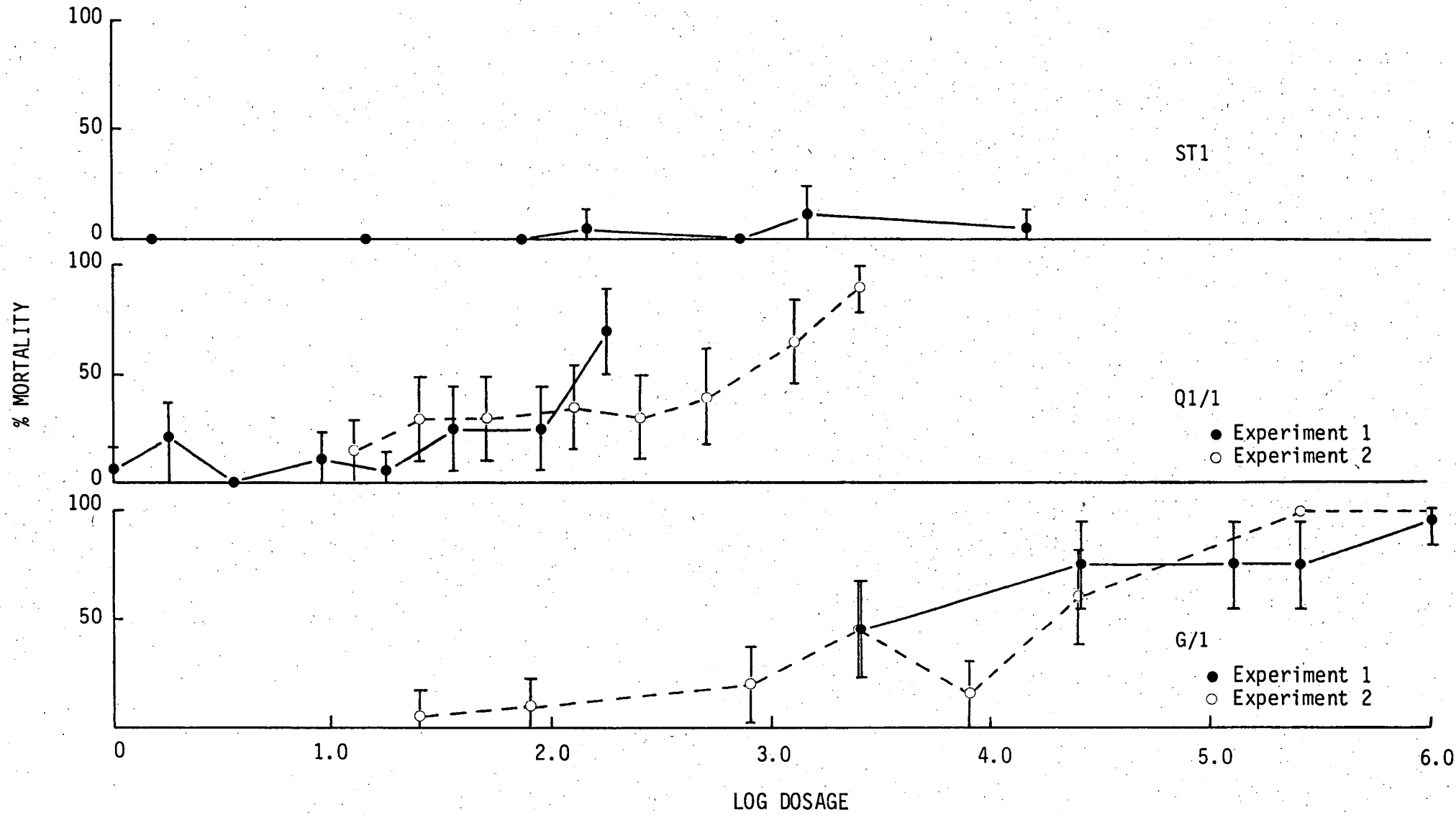

100
50
0
ST1
100
50
0
Q1/1
● Experiment 1
○ Experiment 2
100
50
G/1
● Experiment 1
○ Experiment 2
% MORTALITY
0
1.0
2.0
3.0
4.0
5.0
6.0
LOG DOSAGE

Table 18. Pathogenicity of bacteria associated with nematodes when injected intrahaemocoelically into *Galleria* larvae.

Bacterial Isolate	Nematode Associate	LD_{50}	95% confidence interval
T231	*N. bibionis*	1.5	1.3-2.4
A23	*N. feltiae*	2.0	1.3-2.9
A24		3.0	2.0-4.3
Q385/1	*Neoaplectana* sp. M	-[a]	-
T171		11	3-34
N37	*Neoaplectana* sp. N	4	19
G/1	*N. glaseri*	4,700	360-15,800
		9,700	4,900-22,700
Q/1	Undescribed Steinernematid Q1	360	190-860
SK2	*S. kraussei*	17	9-31
ST1[b]		-[c]	-
B/1	*H. bacteriophora*	20	11-36
T301	*Heterorhabditis* sp., T301	-[a]	-

[a] Not calculated - at all dosages mortality exceeded 50%.

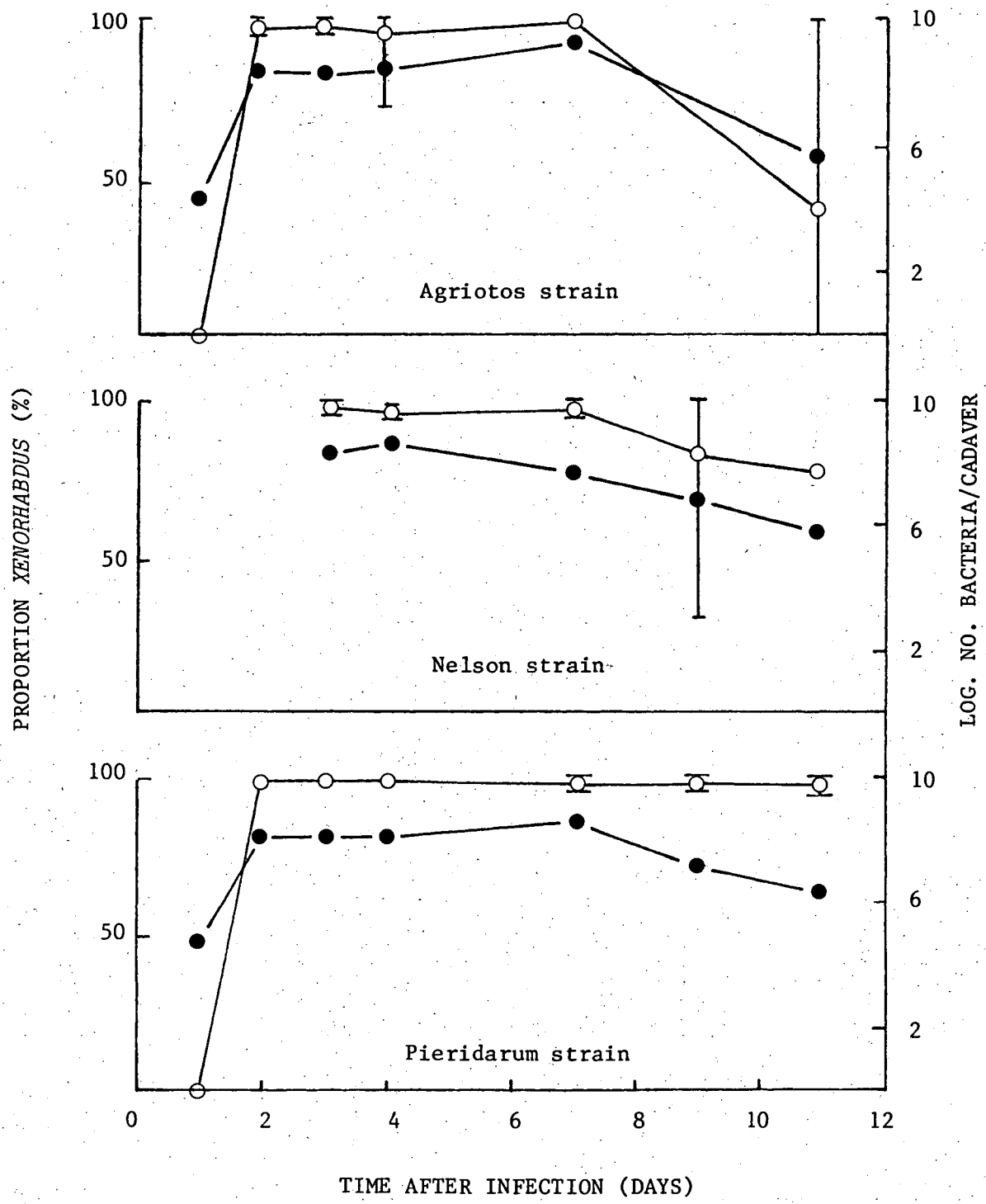
[b] A *Flavobacterium* identified by Mracek (1979) as symbotically associated with *S. kraussei*.

[c] Not calculated - even at an estimated dosage of 14,125 bacteria/larvae, this isolate did not cause >10% mortality.

Table 19. Effect of axenic *N. glaseri* infectives on the pathogenicity of *Xenorhabdus* isolate G/2 injected intrahaemocoelically into groups of 20 *Galleria* larvae.

No. bacterial cells injected	No. dead *Galleria* 3 d after injection: No. axenic *N. glaseri* injected		
	0	1	2
0	0	0	3
115	1	15	19
380	0	17	20
1150	1	18	17

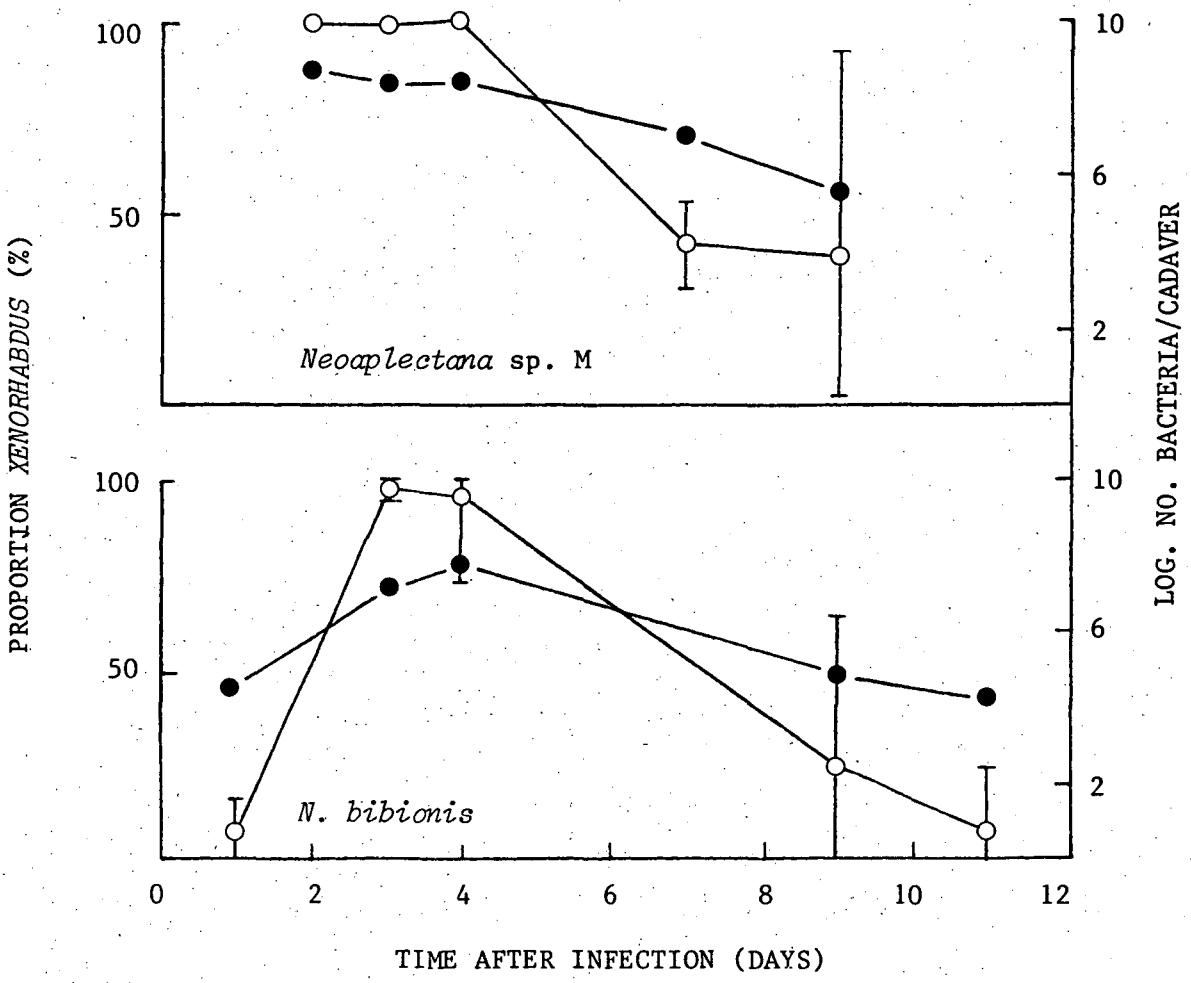
Fig. 13 Proportion of *Xenorhabdus* in the microbial flora of *G. mellonella* larvae infected with *N. feltiae*[a].



[a] ○ % *Xenorhabdus* in bacterial flora. Vertical lines represent S.E.

● Estimated number of bacteria per cadaver.

Fig. 14 Proportion of *Xenorhabdus* in the microbial flora of *G. mellonella* larvae infected with *Neoaplectana* sp. M or *N. bibionis*[a].



[a] ○ % *Xenorhabdus* in bacterial flora. Vertical lines represent S.E.

● Estimated number of bacteria per cadaver.

When infective stage *N. feltiae* carried only secondary form symbiont, this bacterium was unable to dominate the bacterial flora of the *G. mellonella* cadavers as frequently as did the primary form. However, the secondary form symbiont did form the greater part of the bacterial flora of most cadavers after 3 and 6 days (Table 20).

Table 20. Bacterial flora of *Galleria* larvae infected in soil with *N. feltiae* Agriotos infectives[a].

Time after infection (days)	% *Xenorhabdus* in bacterial flora of *Galleria* infected with *N. feltiae* (S.E.)	
	Primary form	Secondary form
3	99.98 (0.009)	99.5 (0.8)
6	99.997 (0.003)	85.4 (27.8)[b]

[a] *Galleria were infected with N. feltiae* Agriotos containing either primary or secondary form bacteria. Four cadavers from each group were sampled at 3 and at 6 days; the haemocoel contents were serially diluted with sterile Ringers solution and spread on NA, NBTA, SSA and DCA. Estimates of the number of *Xenorhabdus* or of other bacteria were made from the agar giving the highest values. The proportion of *Xenorhabdus* in each cadaver was calculated as a percentage and the mean value for the four cadavers calculated after arcsin $\sqrt{P}$ transformation.

[b] Only two cadavers sampled; one was 100% *Xenorhabdus*, the other 50%.

DISCUSSION

The present investigation has shown or confirmed for each known species of Steinernematidae and Heterorhabditidae that there is a specific nematode/bacterium association and that the infective stage of the nematode carries only its symbiont within its intestine. Although the bacteria associated with various species of the Steinernematidae and Heterorhabditidae can be separated taxonomically, they can all be classified within the same genus, *Xenorhabdus*.

All the symbiotic bacteria share a characteristic that has been shown to be important to the nematodes: the ability to make a variety of substrates suitable for the nematodes to feed upon. All but one of the symbionts also share the characteristic of producing two forms, of which the form normally occurring in the infective stage nematode provides better conditions for nematode growth and reproduction.

The importance of these nematode/bacterium interactions is indicated by: the widespread occurrence of the nematode/bacterium associations (Table 1; Thomas & Poinar 1979); the protective action of the nematode (Table 18; Götz *et al.* 1981); the inability of *Xenorhabdus* to survive in soil or water (Poinar 1979) or infect insects when applied *per os* or topically (Poinar & Thomas 1967; Milstead 1979b); the antimicrobial (Tables 7,12; Poinar Hess & Thomas 1980; Paul *et al.* 1981) and nutritive (Poinar & Thomas 1966; Poinar 1979; Bedding 1981) functions of the bacteria. The associations are clearly mutualistic and in nature probably obligatorily mutualistic. The bacteria need the nematodes as a vector and although axenic nematodes of some species can kill their insect host, they fail to reproduce or reproduce very poorly within the host.

The mutualistic nature of the nematode/bacterium association has not, however, been recognized by Lysenko and Weiser (1974) and Lysenko (1979, 1981). Although they isolated many species of bacteria from the nematodes or from infected *G. mellonella* larvae, these workers were unable to isolate *Xenorhabdus*. They concluded that the death of the insects was not merely due to the introduction of *Xenorhabdus* by the nematode but that other, undefined, factors were involved. These results and conclusions are contradicted by a number of other studies. *Xenorhabdus* spp. have been isolated from the intestine of infective stage steinernematids and heterorhabditids and from the haemocoel of nematode-infected insects in the present investigation (Table 1) and in many other studies (Poinar & Thomas 1966; Khan & Brooks 1977; Poinar *et al.* 1977; Poinar & Brooks 1977; Thomas & Poinar 1979; Milstead 1979b; Wouts 1979; Götz *et al.* 1981). The present investigation also showed that *Xenorhabdus* overwhelmingly dominated the flora of *G. mellonella* larvae while the nematodes were reproducing (Figs. 13,14). The failures of Lysenko & Weiser (1974) and Lysenko (1979) to detect *Xenorhabdus* may have been due to their sampling non-infective stages of *N. feltiae* at a time when the insect cadaver had been secondarily colonised by other microorganisms. It is also possible that the cadavers were heavily contaminated. Heavy contamination of cadavers can result from infection by large numbers of infective stage nematodes carrying contaminating bacteria on their external surfaces (Poinar 1979).

S. kraussei is the only species of the Steinernematidae or Heterorhabditidae reported to be symbiotically associated with a bacterium that was not a *Xenorhabdus* sp. The bacterium isolated from

sawfly larvae infected with *S. kraussei* and reported to be symbiotically associated with *S. kraussei* was identified by Mráček (1977) as a *Flavobacterium* sp.

The taxonomic study described in this thesis confirms Mráček's identification. However, the bacteria later isolated from infective stage *S. kraussei* collected from the field were very similar to those isolated from *N. bibionis* (Fig. 3) and identified by Thomas & Poinar (1979) as *X. nematophilus*. Examination of the specificity of the nematode/bacterium association in this investigation showed that the *Flavobacterium* sp. did not have the same relationship to *S. kraussei* as did the *Xenorhabdus*. This latter bacterium is transported from cadaver to fresh host in a specialised organ of the infective juvenile's intestine; it was demonstrated that the *Flavobacterium* was not so carried. The ability of the nematode to grow and reproduce in monoxenic culture with the *Flavobacterium* sp. was of little significance since nematode fecundity in these cultures was much lower than in cultures with the *Xenorhabdus* and several *Neoaplectana* spp. could also be cultured monoxenically with this *Flavobacterium* sp. (Table 13). Moreover, the *Xenorhabdus* sp. was highly pathogenic for insects and able to inhibit the growth of other microorganisms while the *Flavobacterium* sp. was not (Table 7,17). Consequently, the *Xenorhabdus* must be recognized as the symbiont of *S. kraussei* and the *Flavobacterium* sp. as an incidental associate.

Thomas & Poinar (1979) considered that the symbionts of *Heterorhabditis* and those of *Neoaplectana* spp. constituted two species of the same genus. However, analysis of the data gathered in the

taxonomic study described here showed the symbionts of *Heterorhabditis* spp. to be more similar to the *Flavobacterium* sp. of Mráček (1977) than to the symbionts of *Neoaplectana* spp. (Figs. 2,3). Since the results of the sorting strategy used were affected by group size, and only two (identical) isolates of the *Flavobacterium* sp. were studied, the separation of *X. luminescens* and *X. nematophilus* into different genera was not considered to be warranted. The inability of *Neoaplectana* spp. to grow in association with the symbionts of *Heterorhabditis* spp. in *in vitro* monoxenic culture (Table 13) does not support a generic separation because *Neoaplectana* were also unable to utilise the symbiont of the steinernematid Q1 (which clustered with the *Neoaplectana* symbionts; Figs. 2,3). On the other hand, the common abilities of the two species to produce antimicrobial agents, to be carried within an infective stage nematode and to be highly pathogenic for insects do not necessitate their inclusion within a single genus. These characteristics are more or less essential in the niche occupied by the bacteria and could therefore result from convergent evolution. That the two species are related however, is indicated by their similar DNA base composition (Table 9; Thomas & Poinar 1979; Khan & Brooks 1977) and the occurrence of two forms. Clearly the relationship between these two species is uncertain and requires more detailed examination with cell wall analyses, DNA/DNA and/or DNA/RNA hybridisation or at least a taxonomic study that would include related genera of bacteria that are not symbiotically associated with nematodes. Until further evidence becomes available, the two species, *X. luminescens* and *X. nematophilus*, should be left within one genus.

Analyses of the taxonomic data showed clustering of the symbionts of *Heterorhabditis* spp. with no logical subdivision of the group (Group B, Fig. 2; Group H, Fig. 3). In both analyses (i.e. including and excluding the three characters occurring in the primary form only of the symbionts of the Steinernematidae), the two forms of the symbiont of the Polish strain of *Heterorhabditis* were placed in different sub-groups (Figs. 2,3).

In the analyses of the full data set, the symbionts of the Steinernematidae fall into four major groups. Group C contained the primary form symbionts of *S. kraussei* and some *N. bibionis* symbionts while Group D contained the secondary form *S. kraussei* symbiont, the remaining *N. bibionis* symbionts, and those of *N. feltiae* and the steinernematid Q1. However, within Group D the two secondary form *N. feltiae* symbionts clustered more closely to the symbionts of *N. bibionis* and Q1 than to the primary form from which they derived. The primary form symbionts of *Neoaplectana* sp. M comprised Group E while the secondary forms of the symbionts of this nematode were clustered in Group F with the symbionts of *Neoaplectana* sp. N and of *N. glaseri* (the last of which does not have a primary form like those of the other symbionts, Tables 10,12). This clustering resulted in the separation of the secondary forms of the symbionts of the Steinernematidae and the primary forms from which they arose. This indicated that those characteristics that were shown to be peculiar to the primary forms of the symbionts of the Steinernematidae should be excluded from the analysis. When this was done bacteria associated with any one nematode species were clustered together (Group I, the symbionts of *S. kraussei*

and *N. bibionis*; Group J, those of *N. feltiae*; Group K, those of *Neoaplectana* species M and N; Group L, those of *N. glaseri* and the steinernematid Q1; Fig. 3).

In neither analysis was the sub group of the two isolates of the symbiont of *N. glaseri* tightly clustered with any other sub-group, despite its small size and the use of a group-size dependent sorting strategy. The symbiont of *N. glaseri* can, therefore, be seen to fall outside the other groups of steinernematid symbionts. The grouping of the symbionts of *N. glaseri* and of the steinernematid Q1 in the second analysis may, however, be misleading. In the analysis of the complete data set, both forms of the Q1 symbiont were clustered very closely in Group D while the two isolates of the *N. glaseri* symbiont were clustered together in Group F. The small number of isolates of these two symbionts examined prevents a reliable assessment of their relationship.

The bacterial symbionts of the Steinernematidae can be seen to fall into five groups, designated I, J, K, L_1 (the symbionts of *N. glaseri*) and L_2 (the symbionts of Q1)(Fig. 3). The relationship of Group L_2 to any or all of the others must be considered indeterminate.

All members of Groups I, J, K and L_1 lacked catalase, cytochrome oxidase and peroxidase activity. The characteristics that distinguish Groups I, J, K and L_1 from each other are presently considered insufficient to warrant their being placed in separate species. However, the association of each group with particular nematode species may warrant taxonomic separation. Since the members of these four groups can be readily distinguished from each other, they should be considered sub-species rather than merely strains or varieties. Details of the proposed sub-species are presented in Appendix III.

Results obtained from some of the tests used in this taxonomic study differ from those reported by Thomas & Poinar (1979) and Khan & Brooks (1977) in the following respects: the production of two colony forms, a characteristic of all symbionts studied that had not been previously recognized; the bacterial symbionts examined in the present investigation produced acid from some but not all carbohydrates tested; none of the isolates produced glutamic acid decarboxylase; some produced phenylalanine deaminase and/or urease; all produced lipase on egg yolk agar; some did not form blue colonies on tergitol-7 + TTC; the G + C content of the DNA was outside the range used by Thomas & Poinar (1979) to define the genus *Xenorhabdus*.

Some of these differences may be attributed to differences in the methods used. Bromocresol purple was used in the present investigation for the study of acid production because *Xenorhabdus* isolates produced enough acid from the basal medium to affect bromothymol blue or phenol red, used by Thomas & Poinar (1979) and Khan & Brooks (1977) respectively. (Thomas & Poinar (1979) also used bromocresol purple but may have been influenced in their interpretation of colour change by changes seen when using bromothymol blue). Phenylalanine deaminase production was tested at 5 and 7 days as well as 2 days as prescribed in the Difco Laboratories (1968) because results obtained at 2 days were inconsistent. Citrate utilization was tested on OY agar rather than on Simmons' Citrate Agar because the isolates grew poorly on the latter. Lipase activity was tested with fresh egg yolk emulsion rather than with egg yolk extract. The methods used in this study to detect urease and the utilization of organic acids may also have been different from those of Thomas & Poinar (1979), which were not specified.

There must be some doubt about the range of G + C content of DNA of *Xenorhabdus* spp. Thomas & Poinar (1979), in describing the genus, quote values of 43-44 mol% for nine isolates, Khan & Brooks (1977) recorded 45.6 mol% for one of those isolates, and in this study, values ranged from 46.9-50.0 mol% for another seven isolates. Khan & Brooks (1977) estimated the G + C content by a thermal denaturation method. Although Thomas & Poinar (1979) did not specify the method used to obtain their estimates, some estimates were provided by Dr. M. Mandel and were almost certainly obtained by measurement of bouyant density. The differences in G + C content of strain NC19 reported by Khan & Brooks (1977) and Thomas & Poinar (1979) may only reflect differences in methods of determination. The differences in estimates of G + C content reported by Thomas & Poinar (1979) and those reported here may be due to the calculation of (G + C) content from bouyant density. Skyring & Jones (1972) showed differences of up to 4.8% for calculations by the formulae of Schildkraut *et al.* (1962) and of Saunders *et al.* (1964). Unfortunately, Poinar & Thomas (1979) did not provide data on bouyant density values or specify the formula used to calculate (G + C) content. Clearly this aspect of the genus description requires further examination.

The results of the taxonomic study reported here indicate that the genus *Xenorhabdus* and the species *X. nematophilus* and *X. luminescens* and the four sub-species of *X. nematophilus* be recognized. Amended descriptions of the existing taxons and descriptions of the proposed sub-species are presented in Appendix III.

The existence of two forms of a species of bacterium, such as was found for *Xenorhabdus*, does not constitute a unique phenomenon. Examples of colony variation have been reported for other bacteria (e.g. *Ps. aeruginosa*, Zierdt & Schmidt 1964; *Neisseria gonnorrhoeae*, Swanson 1978; *E. coli*, Eisenstein 1981) and even the *Flavobacterium* sp. previously thought to be symbiotically associated with *S. kraussei* produced two colony types. However, the occurrence of this phenomenon in all the symbionts of the Steinernematidae and Heterorhabditidae indicates that it may be significant in he nematode/bacterium interaction.

The primary form of *Xenorhabdus* was originally defined on the basis of its natural occurrence in the infective stage nematode (Akhurst 1980). However, it is perhaps better defined in terms of those characteristics separating it from the secondary form. The primary form is, therefore, re-defined as follows: the primary form of *Xenorhabdus* is the form normally isolated from infective stage nematodes; it produces antimicrobial substances; it allows faster and significantly greater reproduction by the nematode than does the secondary form; it forms blue colonies on agar media containing BTB; it forms narrower but higher, more intensely pigmented colonies than does the secondary form; it is unstable, changing to a secondary form. This re-definition excludes both isolates of the *N. glaseri* symbiont examined in this study as neither possessed all of these characteristics. It does not, however, exclude the possibility that a primary form of this sub-species exists; although none of the bacteria isolated from Tasmanian populations of *Neoaplectana* species M fitted the description of the

redefined primary form, bacteria from each population of this species in Queensland did.

The significance of dimorphism in *Xenorhabdus* to the nematode/bacterium interaction is not clear because the secondary form does not always occur in infected insects and has no clear function. It may have no current significance but may simply be the evolutionary predecessor of the primary form.

The predominance of *X. nematophilus* in *G. mellonella* infected with *N. feltiae* carrying only secondary form symbionts (Table 20) and the continued existence of strains of *N. glaseri* and *Neoaplectana* sp. M that apparently have only the secondary form symbiont, demonstrate that this form is adequate to ensure the survival of the nematode/bacterium association. If the primary form evolved from the secondary, selective pressure may have resulted in the nematodes' ability to preferentially retain the former thus ensuring their continued association with this form that increases reproductive rate and substantially reduces competition for the cadaver by microorganisms. The survival of the secondary form alongside the primary can for the present only be explained by the demonstrated instability of the latter.

Although the nature of the primary form is similar throughout the genus *Xenorhabdus*, there are some inter- and intra-specific variations. When *X. nematophilus* strains change from primary to secondary form, they lose lecithinase activity along with the primary characteristics; *X. luminescens* retains lecithinase activity in both forms. *X. nematophilus* sub-species No. 1 gains two characters (lipase, phenylalanine deaminase) when it loses its primary characteristics.

X. nematophilus subspecies No. 3 not only loses lecithinase but also the ability to hydrolyse casein when it changes to the secondary form. The stability of the primary from varied considerably even within sub-species. Although the secondary form of most isolates was stable under the conditions used, secondary form isolates of two sub-species of *X. nematophilus* were unstable in broth and anaerobic cultures. The variability in the expression of the dimorphism phenomenon was not so great that more than one basic mechanism for form change in *Xenorhabdus* needs to be postulated.

It seems that the determinant of form change in *Xenorhabdus* is neither a plasmid nor lysogenic bacteriophage as primary or secondary form characteristics do not appear to be transferable in mixed cultures and attempts to demonstrate the presence of bacteriophage or plasmids by the use of mutagens and by physical identification methods were unsuccessful. The slight change of form that occurred in broth containing sodium dodecyl sulphate was not necessarily due to "curing". Salisbury *et al.* (1972) and Tomoeda *et al.* (1974) consider that the "curing" action of sodium dodecyl sulphate is due to the greater sensitivity of piliated cells and that it acts as a selective agent. Consequently its limited success in promoting the change from secondary form to primary form may have been due to selection of the primary form which had been produced in the culture by some means other than elimination of a plasmid.

Studies on the phenomenon of reversible form change in other genera of bacteria have not given any strong indication of the nature of the mechanism that determines form change in *Xenorhabdus*. In

X. nematophilus, change of form occurs at very high rates (> 20%, Table 11), occurs in both directions under essentially the same conditions (unshaken broth cultures), and apparently does not occur until the stationary phase is reached. The last two characteristics exclude the Govan *et al.* (1979) model for *Ps. aeruginosa* (spontaneous mutation followed by selection for the mutant). This model is also excluded by the inability of mutagens to enhance the form change in *Xenorhabdus*. Flagellar phase variation in *E. coli* (Eisenstein 1981) occurs during the growth phase and at frequencies of only 2.8% and 3.0% respectively. Although phase variation in *N. gonorrhoeae* occurs largely after the growth phase (Norlander *et al.* 1979), its frequency is much lower (<2.1%) than in *X. nematophilus*. Moreover phase variation in *N. gonorrhoeae* was related to autolysis and decline in cell numbers (Norlander *et al.* 1979) unlike the form change of *X. nematophilus* that may occur when cell numbers are stable. Starlinger and Saedler (1976) found that polar mutations with a greater than normal degree of polarity and whose reversion rate was not enhanced by mutagens were due to insertion sequence (IS) elements. The change of form of *Xenorhabdus* and resultant gain or loss of primary form characteristics was also unaffected by mutagens and may be influenced by insertion/excision of an IS-element or transposon. No conclusion on the mechanism of form change in *Xenorhabdus* was reached in the present investigation.

The antimicrobial activity of primary form *Xenorhabdus* was much wider than had been previously shown. The ability of *Xenorhabdus* to inhibit yeasts and actinomycetes as well as Gram positive and Gram negative bacteria is important to the nematode/bacterium association

because members of all these groups are potential competitors for the insect cadaver. The bioluminescence of *X. luminescens* may also be a means of excluding competitors. Because soil dwelling organisms are more likely to be repelled than attracted by light, bioluminescence may deter saprophagic arthropods and/or other nematodes from destroying the cadaver before the *Heterorhabditis* can complete their life cycle.

The production of antimicrobials by primary form *Xenorhabdus* does not exclude all other microorganisms from the cadaver. However, except on the day immediately following infection when there are few bacteria of any species in the insect, *Xenorhabdus* overwhelmingly dominated the flora until the nematodes had completed reproduction and were forming the infective stage (Figs. 13,14). When secondary form was introduced into the insect instead of primary form, it did not overwhelmingly dominate the flora in all cadavers (Table 20). Consequently nematodes carrying secondary form symbiont (e.g. *N. glaseri*) would not be expected to reproduce in every insect that they infected because some cadavers would putrefy.

The spectrum of antimicrobial activity varied within and between *Xenorhabdus* species with no two primary form isolates having the same spectrum (Table 13). For any pair of isolates, except T319/1 and SK3/1, there was at least one organism sensitive to one and not the second and another organism sensitive to the second but not the first (e.g. *Sh. sonnei* was sensitive to A24 but not NC/1 while the *Flavobacterium* sp. was sensitive to C/1 but not A24). Moreover, some *Xenorhabdus* strains were mutually inhibitory (T319/1 and C/1, SK3/1 and C/1). This variation indicates that, except possibly T319/1 and SK3/1 which may

only vary quantitatively, each of the *Xenorhabdus* strains produced a different combination of antimicrobial agents.

Paul *et al.* (1981) isolated completely different classes of antibacterial compounds from *X. nematophilus* and *X. luminescens*. Identification of antimicrobial agents and their biosynthetic pathways might provide useful information for clarifying the taxonomy of *Xenorhabdus*.

Poinar, Hess & Thomas (1980) detected in cultures of *X. nematophilus* and *X. luminescens* phage tail-like particles that they identified as defective bacteriophages. They found that these particles attached to *B. cereus* cells and concluded that they were identical with the bactericidal agent. As distantly related bacteria may adsorb a phage whose multiplication they do not support (Luria & Darnell 1967), the attachment of these particles to cells of a sensitive bacterium does not necessarily demonstrate a connection with bacterial action. The particles detected by Poinar, Hess & Thomas (1980) could possibly be bacteriocins, some of which have the form of phage tails (Bradley 1967). Bacteriocins normally affect only bacteria closely related to their producer (Reeves 1972) and so the particles detected by Poinar, Hess & Thomas (1980) may be responsible for the inhibition of other *Xenorhabdus* isolates.

The specificity of a nematode/bacterium association is determined not only by the ability of the bacterium to provide suitable nutrient conditions but ultimately by its ability to lodge in the intestine of the infective stage nematode. While bacteria from several genera were able to provide at least partially suitable nutrient conditions for

Neoaplectana spp. (Table 13; Poinar & Thomas 1966; Poinar 1979), the only bacteria able to lodge in the intestine of most infective nematodes were those isolated from that nematode species (Table 15; Poinar 1979).

The degree of specificity of the nematode/bacterium associations varied. While infective stage *N. feltiae* were unable to carry the symbiont of any other species, infective stages of *N. bibionis* and *Neoaplectana* sp. M were almost as efficient in carrying the symbionts of *S. kraussei* and *Neoaplectana* sp. N respectively as they were in carrying their own symbionts. It is, however, notable that *Neoaplectana* sp. N was much less able to carry the symbiont of species M than its own symbiont.

The proportion of *N. glaseri* and *Neoaplectana* sp. M that carry their respective symbionts is lower than that of the other *Neoaplectana* species (Table 15). The low proportion of infective juveniles containing bacteria could result in the inability of the nematodes to reproduce in a host insect because of a failure to introduce the symbiont. Since there appears to be no advantage to *N. glaseri* or *Neoaplectana* sp. M in having bacteria in only half the population, these nematode/bacterium associations seem to be marginally less efficient than the associations between other *Neoaplectana* spp. and their symbionts. *N. glaseri* and the Tasmanian populations of *Neoaplectana* sp. M appear to be at a further disadvantage by being associated with the secondary form of their symbionts. The association between *N. glaseri* and *Neoaplectana* sp. M and their respective symbionts might be considered to be evolutionarily primitive.

The reason for the large variation in the proportion of infective stage *N. glaseri* that contain symbionts (Table 16) is uncertain. It seems likely that this variation was due to the variability in quantity of the symbiont available when infective juveniles were produced because even in monoxenic *in vitro* culture, the symbiont may be almost undetectable at the time of formation of infective juveniles (Bedding *pers. comm.*). Similarly the variation in the proportion of large and small infective stage *N. glaseri* may be due to temporal or spatial variation in the distribution of the symbiont. At times when, or in local areas where, the symbiont is abundant, nutrient conditions may favour the formation of large juveniles but when or where the symbiont is absent, poor nutrient conditions may lead to the formation of small juveniles.

Most *Xenorhabdus* isolates tested were highly pathogenic when injected intrahaemocoelically into *G. mellonella* larvae (Table 17). While the LD_{50} of only two of the isolates tested (the symbionts of *N. glaseri* and the undescribed steinernematid Q1) were higher than the range (5-100 cells) given by Lysenko (1981) for facultative, non-sporeforming pathogens injected into lepidopterous larvae, the LD_{50} values of six isolates were actually below this range.

A low LD_{50} value does not appear to be very important when the nematodes infect lepidopterous insects. *N. feltiae* (Götz *et al.* 1981) and *N. glaseri* (Table 18) have been shown to act synergistically with their symbionts to kill *H. cecropia* and *G. mellonella* respectively. The highly pathogenic nature of most of the symbiotic bacteria may be important in insects of other groups in which the nematodes are unable

or less able to protect their symbionts from the insect's antibacterial defenses. The high level of pathogenicity of *Xenorhabdus* for *G. mellonella* does not demonstrate a high level of pathogenicity for all insects; Götz *et al.* (1981) found an LD_{50} of 500 for *X. nematophilus* injected into *H. cecropia* pupae. In evolving the ability to kill a wide range of insect hosts, *Xenorhabdus* spp. may have become more pathogenic than necessary to kill *G. mellonella*.

This study has explored some of the important features of the symbiotic associations between insect pathogenic nematodes and bacteria. It has confirmed that the associations are mutualistic and shown that the mutualism is probably obligatory in the natural state. The presence of two forms of the bacterial symbionts has been shown and their significance examined. The taxonomy of the bacterial symbionts has been more extensively examined than previously. There remain, however, many interesting aspects of the nematode/bacterium associations that have not been fully examined. In particular, the mechanism that enables the infective stage nematode to retain its symbiont selectively and the nature of the antimicrobial agents and their action *in vivo* have yet to be determined. The nature of the nutrients produced for the nematodes by bacterial metabolism is unknown. The reason why some nematode species are unable to utilize the metabolic products of the bacterial symbiotic with another species (e.g. *N. feltiae* and the symbiont of *N. bibionis*) is also unknown. Is this inability due to the underproduction of an essential nutrient factor, production of a toxic inhibitory compound, or both? The relationships of *X. luminescens* to *X. nematophilus* and that of the symbiont of the steinernematid Q1 to

other *Xenorhabdus* (and of Q1 to other steinernematids) as well as the relationship of *Xenorhabdus* to other Enterobacteriaceae remain uncertain and elucidation of these relationships requires a major taxonomic study. One of the more interesting aspects of the nematode/bacterium associations was the phenomenon of reversible change of form. No explanation of the underlying mechanism for this can be given without a more detailed examination of its genetic basis. Since this phenomenon appears to differ from similar phenomenon in other genera of bacteria, its study might lead to a significant contribution to the understanding of basic genetic mechanisms.

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APPENDIX I

Publications Arising From Studies Described In This Thesis

AKHURST, R.J. AND BEDDING, R.A. 1978. A simple cross-breeding technique to facilitate species determination in the genus *Neoaplectana*. *Nematologica* 24, 328-330.

AKHURST, R.J. 1980. Morphological and functional dimporphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* 121, 303-309.

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AKHURST, R.J. 1982. A *Xenorhabdus* sp. (Eubacteriales : Enterobacteriaceae) symbiotically associated with *Steinernema kraussei* (Nematoda : Steinernematidae). *Revue de Nematologie* 5 (in press).

AKHURST, R.J. 1983. Taxonomic study of *Xenorhabdus* a genus of bacteria symbiotically associated with insect pathogenic nematodes. *International Journal of Systematic Bacteriology* 33 (in press).

APPENDIX II

Full details of data used to examine the taxonomy of bacterial symbionts of the Steinernematidae and Heterorhabditidae.

Characteristics common to all isolates[a]

Characteristic	Result[b]	Characteristic	Result
Gram stain	-	DNase	+
Cell morphology	Rod	Potato starch hydrolysis	-
Acetoin	-	Soluble starch hydrolysis	-
Methyl red	-	Chitinase	-
Lysine decarboxylase	-	KCN tolerance	+
Ornithine decarboxylase	-	Protease - Loeffler's medium	-
Glutamic acid decarboxylase	-	egg albumen agar	-
Arginine dihydrolase	-	Tyrosinase	-
Production of gas from		Lipase - egg yolk agar	+
adonitol	-	MacConkey agar (growth on)	+
aesculin	-	Simmons nitrate agar	n
arabinose	-	Utilisation (in Shaw & Clark's medium) of gluconate	-
galactose	-	Utilisation (in OY medium) of	
inulin	-	acetate	+
lactose	-	benzoate	-
mannitol	-	citrate	+
melibiose	-	fumarate	+
β-methyl-glucoside	-	malate	+
raffinose	-	oxalate	-
rhamnose	-	succinate	+
sorbose	-	tartrate	-

[a] See Appendix II for list of isolates.

[b] Symbols: +, positive; -, negative; n, no growth.

CHARACTERISTIC	B/1	C/1	NZH	D/1	HP/1	HP/2	Q380	V16	T280/1	T280/2	T301	T310	T327
Host nematode[1]	Hb	Hb	Hb	H	H	H	H	H	H	H	H	H	H
Mean cell length (μ)	6.7	6.0	6.0	5.4	6.2	6.4	4.8	4.8	4.8	5.9	6.8	4.7	6.0
s.e.	2.1	2.0	2.1	2.0	3.6	3.3	2.2	3.0	1.7	2.5	2.9	1.4	2.2
Mean cell width (μ)	1.0	1.0	1.3	1.1	1.1	1.2	1.1	1.0	0.9	1.0	0.9	0.9	1.0
s.e.	0.1	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Flagella[2]	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt
Pigmentation[3]	y-br	y-r	y	bu-r	o-r	y	y-o	p-br	y	y	y-o	o-r	y
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Peroxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Bioluminescence	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Reducing compounds from sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipase (Tween 80)	+	+	+	+	+	+	+	+	+	+	+	+	+
Lecithinase	+	+	+	+	+	+	+	+	+	+	+	+	±
Phenylalanine deaminase	-	+	-	-	+	+	-	-	+	+	-	-	+
Indole	-	-	-	+	-	-	-	-	+	+	-	-	-
Phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+
Antibacterial activity	+	+	+	+	+	-	+	+	+	+	+	+	+
Pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+	+
Absorbtion of BTB	+	+	+	+	+	-	+	+	+	+	+	+	+
MacConkey - red colonies	+	+	+	+	+	-	+	+	+	+	+	+	-
Triple sugar iron agar[4] (slope/butt/H_2S)	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n
Mucoid colonies	+	+	+	+	+	-	+	+	+w	-	+	+	+
Growth at 34°	+	+	-	+	+	-	+	+	-	-	+	-	+
36°	-	-	-	+	-	-	+	-	-	-	-	-	-
38°	-	-	-	+	-	-	+	-	-	-	-	-	-
40°	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	+	+	+	+	+	+	+
gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+
propionate	+	+	+	+	+	+	+	+	+	+	+	+	-
Acid from dextrin	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-
inositol	±	±	±	-	±	±	-	-	±	-	±	±	-
maltose	+w	+	+w	+w	+w	+w	+w	+	+w	+w	+w	+w	+w
mannose	+	+	+	+	+	+	+w	+	+	+	+	+	+
melezitose	+w	+w	+w	±	+w	+w	±	±	+w	+w	+w	+w	+w
α-methyl-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-
ribose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	-
saccharose	-	-	-	-	-	-	-	-	-	-	-	-	-
salicin	-	±	±	-	-	-	-	-	-	-	-	-	-
sorbitol	-	-	-	-	-	-	-	-	±	-	-	-	-
sucrose	-	-	-	±	-	-	-	-	-	-	-	-	-
trehalose	+w	+w	+w	±	+w	+w	±	+w	+w	+w	+w	+w	+w
xylose	-	-	-	-	-	-	-	-	-	-	±	-	-

[1] Hb, *H. bacteriophora*; Hh, *H. heliothidis*; H, *Heterorhabditis*, species uncertain.

[2] Pt, Peritrichous.

[3] br, brown; bu, buff; o, orange; p, pink; r, red; y, yellow.

[4] +, alkaline; -, acid; n, no H_2S.

Other symbols: +, positive; +w, weakly positive; ±, doubtful; -, negative.

CHARACTERISTIC	NBC	N51	N60/1	NZ	T228	T231	T268	T292	T298	T302	T307	T319/1
Host nematode[1]	Nb	Nb	Nb	Nb	Nb	Nb	Nb	Nb	Nb	Nb	Nb	Nb
Mean cell length (µ)	5.2	6.2	4.6	6.3	5.4	6.3	4.8	5.6	5.5	5.5	6.4	6.1
s.e.	2.1	2.4	1.7	2.1	1.7	2.7	1.3	1.7	1.6	1.3	2.0	1.9
Mean cell width (µ)	1.3	1.1	1.2	1.1	1.0	0.9	0.9	0.9	0.9	1.0	1.0	1.0
s.e.	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Flagella[2]	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt
Pigmentation[3]	y	y	y	y	y	y	y	y	y	y	y	y
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Peroxidase	-	-	n[4]	-	-	-	-	-	-	-	-	-
Bioluminescence	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	-	+	+	+	+	+	+	+	+	+
Reducing compounds from sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-
Lipase (Tween 80)	+	+	+	+	+	+	+	+	+	+	+	+
Lecithinase	+	+	+	-	+	+	+	+	+	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	-	-	-	-	-	-	-	-	-	+	-	-
Antibacterial activity	+	+	+	+	+	+	+	+	+	+	+	+
Pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+
Absorbtion of BTB	+	+	+	+	+	+	+	+	+	+	+	+
MacConkey - red colonies	-	-	-	-	-	-	-	-	-	-	-	-
Triple sugar iron agar[5] (slope/butt/H_2S)	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n
Mucoid colonies	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 34°	-	-	-	-	-	-	-	-	-	-	-	-
36°	-	-	-	-	-	-	-	-	-	-	-	-
38°	-	-	-	-	-	-	-	-	-	-	-	-
40°	-	-	-	-	-	-	-	-	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	+	+	+	+	+	+
gluconate	+	+	-	+	+	+	+	+	+	+	+	+
propionate	+	+	+	+	+	+	-	+	+	-	-	-
Acid from dextrin	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
fructose	+	+	+	+	+	+	+	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	-	±	+w	-	+w	±	±	-	±	±	±	-
inositol	±	±	-	±	±	±	±	±	-	±	±	±
maltose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
mannose	+	+	+	+	+	+	+	+	+	+	+	+
melezitose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
α-methyl-glucoside	-	-	-	-	-	-	±	±	±	-	-	-
ribose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
saccharose	-	-	-	±	-	-	-	-	-	±	±	-
salicin	-	-	-	-	-	-	-	-	-	-	-	-
sorbitol	-	-	-	-	-	±	-	±	-	±	-	-
sucrose	-	-	-	-	-	-	-	-	-	-	-	-
trehalose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
xylose	-	-	-	±	-	±	-	±	-	±	±	-

[1] *Neoaplectana bibionis*.

[2] Pt, Peritrichous.

[3] y, yellow.

[4] n, not tested.

[5] +, alkaline; -, acid; n, no H_2S.

Other symbols: +, positive; +w, weakly positive; ±, doubtful; -, negative.

CHARACTERISTIC	T335/1	T335/2	V1	V2	A23	A24	A25	AN/5	N55	P1	TN6	TP7
Host nematode[1]	Nb	Nb	Nb	Nb	Nf	Nf	Nf	Nf	Nf	Nf	Nf	Nf
Mean cell length (μ)	6.1	5.6	6.0	6.4	5.5	5.7	5.2	7.1	6.5	5.8	6.3	6.2
s.e.	2.4	2.2	1.9	1.4	1.8	2.1	1.9	2.3	3.1	3.0	1.7	1.8
Mean cell width (μ)	1.2	1.2	0.9	1.0	1.1	1.2	1.2	1.1	1.0	1.2	1.0	1.0
s.e.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Flagella[2]	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt
Pigmentation[3]	y	y	y	y	bu	bu	bu	bu	bu	bu	bu	bu
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Peroxidase	-	-	-	-	-	-	-	-	-	-	-	-
Bioluminescence	-	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reducing compounds from sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-
Lipase (Tween 80)	+	+	+	+	+	-	+	-	-	-	+	-
Lecithinase	+	-	+	+	-	+	-	+	+	+	+	+
Phenylalanine deaminase	-	±	-	-	+	-	+	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	-	-	-	-	-	-	-	-	-	-	-	-
Antibacterial activity	+	-	+	+	-	+	-	+	+	+	+	+
Pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+
Absorbtion of BTB	+	-	+	+	-	+	-	+	+	+	+	+
MacConkey - red colonies	-	-	-	-	-	-	-	-	-	-	-	-
Triple sugar iron agar[4] (slope/butt/H_2S)	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n
Mucoid colonies	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 34°	-	-	-	-	+	+	+	+	+	+	+	+
36°	-	-	-	-	-	-	-	-	-	-	-	-
38°	-	-	-	-	-	-	-	-	-	-	-	-
40°	-	-	-	-	-	-	-	-	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	+	+	+	+	+	+
gluconate	+	+	+	+	-	-	-	+	-	+	+	+
propionate	+	+	+	+	+	+	+	+	+	+	+	+
Acid from dextrin	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
fructose	+	+	+	+	+	+	+	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	±	±	±	-	-	-	-	-	-	-	-	-
inositol	-	-	±	-	-	-	-	±	±	±	-	±
maltose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
mannose	+	+	+	+	+	+	+	+	+	+	+	+
melezitose	±	±	+w	+w	+w	±	+w	+w	+w	+w	+w	+w
α-methyl-glucoside	-	-	-	-	-	-	-	-	-	-	-	-
ribose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
saccharose	-	-	-	-	-	-	-	-	-	-	-	-
salicin	-	-	-	-	-	-	-	-	-	-	-	-
sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
sucrose	±	±	-	-	±	±	±	-	-	-	-	-
trehalose	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
xylose	-	-	-	-	-	-	-	±	-	-	±	±

[1] Nb, *N. bibionis*; Nf, *N. feltiae*.

[2] Pt, Peritrichous.

[3] y, yellow; bu, buff.

[4] +, alkaline; -, acid; n, no H_2S.

Other symbols: +, positive; +w, weakly positive; ±, doubtful; -, negative.

CHARACTERISTIC	G/1	G/2	Q58/1	Q58/2	Q385/1	Q385/2	Q393	T80	T171	T300	N37
Host nematode[1]	Ng	Ng	Nm	Nm	Nm	Nm	Nm	Nm	Nm	Nm	Nn
Mean cell length (µ)	4.4	4.5	3.9	4.6	4.6	5.7	5.3	6.5	5.7	5.6	4.8
s.e.	1.3	1.7	1.7	1.8	1.4	2.0	1.5	3.0	1.9	1.7	2.0
Mean cell width (µ)	1.1	1.2	1.0	1.0	1.0	1.1	1.1	1.1	1.1	1.0	1.2
s.e.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Motility	+	+	+	+	+	+	+	+	+	+	+
Flagella[2]	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt	Pt
Pigmentation[3]	br	br	br	br	br	br	br	br	br	br	br
Catalase	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-
Peroxidase	-	-	-	-	-	-	-	-	-	-	-
Bioluminescence	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	-	+	-	+	-	+	+	+	+
Reducing compounds from sucrose	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	-	-	+	+	+	+	+	+	+	+	+
Lipase (Tween 80)	+	+	+	+	+	+	+	+	+	+	+
Lecithinase	-	-	+	-	+	-	+	-	-	-	-
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	-	-	+	+	+	+	+	+	+	+	+
Antibacterial activity	-	-	+	-	+	-	+	-	-	-	-
Pathogenicity	-	-	+	+	+	+	+	+	+	+	+
Absorbtion of BTB	-	-	+	-	+	-	+	-	-	-	-
MacConkey - red colonies	+	-	-	-	-	-	-	-	-	-	-
Triple sugar iron agar[4] (slope/butt/H_2S)	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n
Mucoid colonies	-	-	-	-	-	-	-	-	-	-	-
Growth at 34°	+	+	+	+	+	+	+	+	+	+	+
36°	+	+	+	+	+	+	+	-	-	-	+
38°	+	+	+	+	-	-	-	-	-	-	+
40°	+	+	-	-	-	-	-	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	+	+	+	+	+
gluconate	+	+	+	+	+	+	+	+	+	+	+
propionate	+	+	+	+	+	+	+	+	+	+	+
Acid from dextrin	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
fructose	+	+	+	+	+	+	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+	+	+	+
glycerol	+w	+w	-	-	+w	+w	-	-	-	-	+w
inositol	-	±	-	±	±	-	-	±	±	±	±
maltose	+w	+	+	+	+	+	+	+	+	+w	+w
mannose	+w	+	+	+	+	+	+	+	+	+	+
melezitose	±	+w	+w	+w	+w	+w	+w	+w	+w	+w	+w
α-methyl-glucoside	-	-	-	-	-	-	-	-	-	-	-
ribose	+w	+w	-	±	±	±	-	+w	+w	+w	+w
saccharose	-	-	-	-	-	-	-	-	-	-	-
salicin	-	-	-	-	-	-	-	+w	+w	-	+w
sorbitol	-	-	-	-	-	-	-	-	-	-	-
sucrose	-	-	-	-	-	-	-	-	-	-	-
trehalose	+w	+w	+w	+w	+w	+w	+w	+w	+	+w	+w
xylose	-	-	-	-	-	-	-	-	-	±	-

[1] Ng, *Neoaplectana glaseri*; Nm, *Neoaplectana* sp M; Nn, *Neoaplectana* sp N.

[2] Pt, Peritrichous.

[3] br, brown.

[4] +, alkaline; -, acid; n, no H_2S.

Other symbols: +, positive; +w, weakly positive; ± doubtful; -, negative.

CHARACTERISTIC	SK2	SK3/1	SK3/2	SK6	SK8	SK9	SK10	ST1	ST2	Q1/1	Q1/2
Host nematode[1]	Sk	Sk	Sk	Sk	Sk	Sk	Sk	n	n	Q1	Q1
Mean cell length (µ)	6.5	5.3	4.9	4.9	6.1	4.0	6.5	2.7	2.6	4.5	6.2
s.e.	2.4	2.2	1.7	1.4	4.3	1.7	2.4	0.6	0.6	1.5	2.7
Mean cell width (µ)	1.1	1.1	1.0	1.1	1.0	1.1	1.1	0.5	0.5	1.0	1.0
s.e.	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0	0	0.2	0.2
Motility	+	+	+	+	+	+	+	-	-	+	+
Flagella[2]	Pt	Pt	Pt	Pt	Pt	Pt	Pt	n	n	Pt	Pt
Pigmentation[3]	y	y	y	y	y	y	y	y	y	y	y
Catalase	-	-	-	-	-	-	-	+	+	+	+
Oxidase	-	-	-	-	-	-	-	+	+	-	-
Cytochrome oxidase	-	-	-	-	-	-	-	+	+	-	-
Peroxidase	n	n	n	n	n	n	n	+	+	n	n
Bioluminescence	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	-	-	+	+
Hugh & Leifson - open tube	+	+	+	+	+	+	+	+	+	+	+
closed tube	+	+	+	+	+	+	+	-	-	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	-	-	+	+
Casein hydrolysis	+w	+w	+w	+w	+w	+w	+w	-	-	+	+
Reducing compounds from sucrose	-	-	-	-	-	-	-	+	+	-	-
Urease	-	-	-	-	-	-	-	+	+	-	-
Aesculin hydrolysis	-	-	-	-	-	-	-	-	-	+	+
Lipase (Tween 80)	+w	+w	±	+w	+w	+w	+w	+	+	+	+
Lecithinase	+w	+w	-	+w	+w	+w	+w	-	-	+	-
Phenylalanine deaminase	-	-	-	-	-	-	-	+	+	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	-	-	-	-	-	-	-	+	+	-	±
Antibacterial activity	+	+	-	+	+	+	+	-	-	+	-
Pathogenicity	+	+	+	+	+	+	+	-	-	+	+
Absorbtion of BTB	+	+	-	+	+	+	+	-	-	+	-
MacConkey - red colonies	-	-	-	-	-	-	-	-	-	-	-
Triple sugar iron agar[4] (slope/butt/H_2S)	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	+/-/n	0/0/n	0/0/n	+/-/n	+/-/n
Mucoid colonies	-	-	-	-	-	-	-	-	-	-	-
Growth at 34°	-	-	-	-	-	-	-	+	+	+	+
36°	-	-	-	-	-	-	-	-	-	+	+
38°	-	-	-	-	-	-	-	-	-	-	-
40°	-	-	-	-	-	-	-	-	-	-	-
Utilisation of formate	+	+	+	+	+	+	+	-	-	+	+
gluconate	+	+	+	+	+	+	+	+	+	-	-
propionate	+	+	+	+	+	+	+	+	+	+	+
Acid from dextrin	+w	+w	+w	+w	+w	+w	+w	±	±	+w	+w
fructose	+	+	+	+	+	+	+	-	-	+	+
glucose	+	+	+	+	+	+	+	+w	+w	+	+
glycerol	-	-	-	-	-	-	-	-	-	+w	+w
inositol	-	-	-	-	-	-	-	-	-	-	-
maltose	+w	+w	+w	+w	+w	+w	+w	-	-	+w	+
mannose	+	+	+	+	+	+	+w	±	±	+	+
melezitose	+w	+w	±	+w	+w	±	+w	±	±	+w	+w
α-methyl-glucoside	-	-	-	-	-	-	-	-	-	-	-
ribose	+w	+w	-	+w	±	-	-	+w	+w	+w	+w
saccharose	-	-	-	-	-	-	-	-	-	±	±
salicin	-	-	-	-	-	-	-	-	-	-	-
sorbitol	-	-	-	-	-	-	-	-	-	-	-
sucrose	-	-	-	-	-	-	-	±	±	-	-
trehalose	+w	+w	+w	+w	+w	+w	+w	±	±	+w	+w
xylose	-	-	-	-	-	-	-	-	-	-	-

[1] Sk, *Steinernema krausaei*; n, none known; Q1, steinernematid, species Q1.

[2] Pt, Peritrichous; n, none.

[3] y, yellow.

[4] +, alkaline; -, acid; n, no H_2S;

Other symbols: +, positive; +w, weakly positive; ±, doubtful; -, negative.

APPENDIX III

Amended descriptions of the genus *Xenorhabdus* and the species *X. nematophilus* and X. luminescens.

Descriptions of four proposed sub-species of *X. nematophilus*.

Xenorhabdus Thomas & Poinar (Amended description)

Gram-negative, asporogenous, peritrichously flagellated rods, approx. 0.8 - 2.0 μ x 2.0 - 17.0 μ. Motile. In older cultures crystalline inclusions (that are not polybetahydroxybutyrate) are formed within cells. Facultatively anaerobic, chemoorganotrophic. Good growth in and on meat extract and simple peptone media. Metabolism is respiratory and fermentative. Acid, without gas, is produced from the fermentation of glucose, fructose, maltose, mannose, dextrin and trehalose.

The bacteria occur in two forms. The primary form is the one normally carried within the infective juvenile nematode; it produces antimicrobial substances and provides conditions suitable for nematode reproduction *in vivo* and *in vitro*. The primary form is unstable producing the secondary form.

Primary form colonies on nutrient agar are about 1 mm in diameter in 24-48 h at 24° (2-3 mm in 72 h at 28°). They are smooth and moist and either granular in appearance or mucoid; low convex; circular with irregular margins. Pigmentation buff, brown, yellow, red, orange or pink; may or may not be bioluminescent. On agar media containing bromothymol blue (BTB), the primary forms absorb BTB decolorising the agar and forming colonies that are blue or green.

Secondary form colonies are flatter and wider (2.5 - 4.5 mm in diameter in 72 h at 28°); they are more translucent, not mucoid, less pigmented and bioluminescent than the corresponding primary form. They do not absorb BTB from agar media.

Growth is not inhibited by KCN. Most strains do not grow at > 36°. Lysine, ornithine and glutamic acid decarboxylases, arginine dihydrolase, oxidase, cytochrome oxidase and peroxidase are not produced; some strains do not exhibit catalase activity. The methyl red and Vogues-Proskauer tests are negative; nitrates are not reduced to nitrates and H_2S is not produced in triple sugar iron agar. Most hydrolyse casein; starch is not hydrolysed. Chitinase and tyrosinase are not produced; DNase and lipase (egg yolk agar) are produced. Gelatin is liquefied. Citrate is not utilised in Simmons citrate agar or gluconate in Shaw and Clark's (1955) medium; acetate, citrate, formate, fumarate, lactate, malate and succinate but not benzoate, malonate, oxalate or tartrate are utilised in Dye's (1968) OY agar; most strains utilise gluconate and propionate in OY agar.

Most isolates are highly pathogenic when injected intrahaemocoelically into *Galleria* larvae. The G + C content of the DNA is 43 - 50 mol%. The natural habitat of these bacteria is the intestinal lumen of entomogenous nematodes and insects infected with these nematodes.

The type species is *Xenorhabdus nematophilus* (Poinar and Thomas).

Xenorhabdus nematophilus (Poinar and Thomas) (Amended description)

As for the genus except: Colonies are neither mucoid or bioluminescent. Neither catalase nor indole are produced. The primary form produces lecithinase; the secondary does not. Phenylalenine deaminase is produced by the secondary form of some strains.

The natural habitat is the intestinal lumen of nematodes of the family Steinernematidae and in insects infected with these nematodes.

No change of type strain (ATCC 19061) is proposed.

Four sub-species are recognised within the species *X. nematophilus*.

Sub-species No. 1[a] (Group J). As for the descriptions of *X. nematophilus* except: pigmentation buff; primary form does not produce lipase in Sierra's (1959) medium or phenylalanine deaminase while secondary form produces both; acid produced from ribose but not salicin or glycerol; propionate utilized; gluconate utilized in OY agar by some strains.

Associated with *N. feltiae*.

Type strain is ATCC 19061[b].

Sub-species No. 2 (Group I). As *X. nematophilus* except: pigmentation yellow; no growth at 34°; acid produced from ribose but not salicin; some strains produce acid from glycerol; gluconate utilized in OY agar; propionate utilized by some strains.

Associated with *N. bibionis* and *S. kraussei*.

Sub-species No. 3 (Group K). As *X. nematophilus* except: pigmentation brown; some isolates grow at 38°; aesculin hydrolyzed; phosphatase produced; casein hydrolyzed by secondary but not primary form; gluconate and propionate utilized in OY agar; some isolates produce acid from glycerol, ribose and/or salicin.

Associated with undescribed *Neoaplectana* species M and N.

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[a] Rules of nomenclature dictate that this must be named *X. nematophilus* subspecies *nematophilus* and have the same type strain as the species.

[b] Designated AN/5 in this study.

Sub-species No. 3 may not be homogenous. Although the symbiont of *Neoaplectana* sp. N clustered with the symbionts of *Neoaplectana* sp. M, study of the specificity of the nematode/bacterium association revealed some differences: *N. glaseri* could be cultured with the symbionts of *Neoaplectana* sp. M but not with that of *Neoaplectana* sp. N and only 7% of *Neoaplectana* sp. N infective juveniles were able to carry the symbiont of species M (Tables 14,16).

Sub-species No. 4 (Group L_1). As *X. nematophilus* except: pigmentation brown; growth at 40°; gluconate and propionate utilized in OY agar; acid produced from glycerol and ribose but not salicin; not highly pathogenic when injected intrahaemocoelically into *G. mellonella* larvae; one form absorbs dye producing red colonies on MacConkey agar.

Associated with *N. glaseri*.

Since sub-species No. 4 is described from only two isolates, the definition must be considered tentative.

Xenorhabdus luminescens Thomas and Poinar (Amended description)

As for the genus *Xenorhabdus* except: most isolates bioluminescent[a]; pigmentation variable; catalase, urease and phosphatase produced; indole and phenylalanine deaminase variable; some strains absorb dye producing red colonies on MacConkey agar; some strains grow at 38°; gluconate and propionate utilized on OY agar by most strains; acid produced from ribose but not glycerol; primary form colonies are highly mucoid.

Natural habitat is the intestinal lumen of nematodes of the genus *Heterorhabditis* and in insects infected with these nematodes.

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[a] Paul *et al.* (1981) reported a non-bioluminescent strain.

APPENDIX IV

Analysis of variance of data on the effect of axenic *N. glaseri* infectives on the pathogenicity of *Xenorhabdus* isolate G/1.

Analysis of variance of data on the effect of axenic *N. glaseri* infectives on the pathogenicity of *Xenorhabdus* isolate G/1.

A. Full Data Set

Source	Sum of squares	df	Mean square	F	P
Nematode	23.4750	2	11.7375	166.738	<0.001
Bacterium	13.6458	3	4.5486	64.616	<0.001
Nematode x Bacterium	6.4917	6	1.08195	15.370	<0.001
Error	16.0500	228	0.07039		
Total	59.6625	239			

B. Data where >1 nematode was injected with >1 bacterium.

Source of variation	Sum of squares	df	Mean square	F	P
Nematode	0.300	1	0.3000	2.948	n.s.
Bacterium	0.1167	2	0.05835	0.573	n.s.
Nematode x Bacterium	0.35	2	0.1750	1.720	n.s.
Error	11.6	114	0.1075		
Total	12.3667	119			